ALPHA-INTERFERON RESPONSES IN CHILDREN WITH RESPIRATORY SYNCYTIAL VIRUS ASSOCIATED BRONCHIOLITIS

by

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SUMMARY

From October 1985 to March 1986 208 children (128 males, 80 females) were admitted to the Royal Hospital for Sick Children, Glasgow with suspected respiratory infection. This hospital is the largest paediatric centre in the West of Scotland with annual admissions for respiratory infections of 1903 children (79.5%). When comparing the total number of children under study and total number of admissions during the study period, the majority of admissions (45%) were children under one year of age with the highest incidence of respiratory infections in November (30.7%) at the RHSC. In this study RSV infection was much greater in infants from Inner Glasgow predominantly Drumchapel, Castlemilk, Toryglen, High Possil (65.8%), and Clydebank (12.6%) which have a higher proportion of low income families and households with two or more indicators of deprivation. The reverse situation was found in Bearsden/Milngavie and Eastwood where the proportion of RSV infections was 2.7% and 5.4% respectively.

A full history and clinical examination was carried out in each case, and data recorded on a special form suitable for computer analysis. This included admission history, diagnosis classified into Upper Respiratory Tract Infection (URTI), pneumonia,

bronchiolitis, chronic lung disease and others. Clinical parameters such as maximum respiratory rate, time to establish normal respiratory rate, physiotherapy and length of tube feeding were evaluated. Nasopharyngeal secretions (NPS) were collected on admission or first day of symptoms if already admitted, and subsequently repeated on days 3, 5, and 7 when possible. Plastic disposable collecting mucus traps with an attached feeding tube (5fg) were used to obtain the secretions. A portion of the secretions was cultured in bacteriological media and the remainder was transferred to virus transport medium (VTM) in a 1/10 dilution. After centrifugation of the specimen suspension, the supernatant fluid was used for virus isolation, embryonated hen egg inoculations and assay for alpha-interferon (IFN-α). The cell pellet was examined by direct immunofluorescence using a respiratory syncytial virus (RSV) specific monoclonal antibody.

IFN-α levels were determined by a commercially available immunoradiometric assay. The test utilises an ^{125}I labelled highly specific monoclonal antibody (Yok 5/19) to leucocyte IFN-α . Prior to application of the test in the clinical study it was important to determine the assay performance in terms of sensitivity and specificity parameters with special reference to the clinical specimens to be

examined. The reproducibility of the test was evaluated when the mean values for each point of different standard curves were plotted together. A standard curve was used to interpolate IFN-α values for unknown samples of NPS. The variability found between the standard curves using different kits of reagents were similar for each point and varied from 21 to 31%. The sensitivity of IRMA for NPS was also evaluated and the test was able to detect as little as 0.2 IU/ml in a diluted clinical specimen. Experimentation on the specimens was carried out to determine heat stability and lability to trypsin. Using recombinant IFN-α or a pool of specimens containing endogenous IFN-α, treatment with heat caused partial inactivation.

The protein characteristics of IFN-α was confirmed when standard preparations of IFN-α and clinical specimens containing endogenous IFN-α were treated with trypsin for trypsin sensitivity. In these experiments trypsin treatment completely inactivated both recombinant and endogenous IFN-α.

In this study neutralization-blocking experiments were performed in order to demonstrate the specificity of the Yok 5/19 monoclonal antibody in detecting IFN-α. In both cases using standards and specimens, it was possible to neutralize almost 100% of the activity with very little residual IFN-α after

blocking, demonstrating an absolute specificity in this test.

It was clear for these studies that this technique is well devised, reproducible, rapid and sensitive.

A clinical study was carried out to determine RSV and IFN-α status in children with respiratory infections.

The distribution of age in RSV positive and negative cases showed a higher proportion of RSV positive cases from 1 to 6 months of age whereas a higher proportion of RSV negative cases were from six to eight months.

IFN-α positivity was found in a higher proportion of younger children and in those with a longer hospital stay.

A male predominance was found in this study and boys were admitted to hospital in higher numbers, whether RSV positive or negative.

The presence of IFN-α in NPS was statistically significant in cases of pneumonia, whether RSV positive or not, and was found in the majority of NPS from patients with a maximum respiratory rate greater than 60/min. The viral aetiology of RSV negative/IFN-α positive pneumonia was unknown in the majority of cases.

In this study it was possible to demonstrate

IFN-α in the NPS of a relatively high percentage of children (38%) with RSV infection compared to other studies. In RSV positive bronchiolitic patients, IFN-α was present in 65% compared to only 31% in bronchiolitic patients due to other causes. Adenovirus infection in patients studied here was associated with an IFN-α mean level of 167.8 IU/ml. When the RSV positive data on specimens and cases were analysed, it appeared that when IFN-α is induced, its presence is consistently associated with the persistence of the virus in secretions. IFN-α is likely to be present at the first testing on admission in NPS of children who will produce IFN-α during the course of RSV infection.

Of the total population studied, 58.6% were found to be RSV positive/IFN-α positive compared to 41.4% RSV positive/IFN-α negative cases. The study results demonstrated that RSV positive patients were statistically different from those RSV negative patients for most of the clinical parameters analysed.

Although trends in various clinical parameters were seen linking RSV disease severity with IFN-α positivity, the only clinical finding which was shown to be statistically significant with the demonstration of IFN-α in NPS was the duration of tube feeding.

The diagnosis of RSV was demonstrated in 89

cases by the direct immunofluorescence technique when culture results were negative. The remaining 22 cases were positive by culture and by fluorescence. It was observed that RSV positive NPS confirmed only by fluorescence were more likely to be in the low positive IFN-α range of 2-20 IU/ml than culture positive samples which had higher mean IFN-α level.

To obtain a better isolation rate for influenza virus, clinical specimens were also inoculated into embryonated hen eggs but these efforts were unrewarding in the present study.

Streptococcus pneumoniae was the bacterium most frequently isolated in NPS in all the clinical parameters analysed.

This study does not give a clear indication where IFN-α fits into the pathogenesis of RSV infection in association with bronchiolitis but has contributed to the knowledge of this infection. However, the presence of IFN-α was associated with more persistent RSV infection and could be considered to provide additional information on the likely outcome of this disease or prompt the use of specific antiviral chemotherapy.

ABBREVIATIONS

CFT	complement fixation test
CPE	cytopathic effect
CSF	cerebrospinal fluid
DNA	deoxyribonucleic acid
eIFN-α	endogenous alpha-interferon
FITC	fluorescein isothiocyanate
GGHB	Greater Glasgow Health Board
HA	haemagglutinin
Hela	cervical carcinoma
HEp-2	continuous human epithelioma cells
HuIFN-α	human alpha-interferon
HuIFN-β	human beta-interferon
HuIFN-γ	human gamma-interferon
^{125}I	iodine 125
IFN	interferon
IFN-α	alpha-interferon
IgA,E,G,M	immunoglobulins A, E, G and M
IRMA	immunoradiometric assay
IU/ml	units of interferon with respect to the appropriate international reference standard
MEM	eagle's minimum essential medium
mIFA	monoclonal fluorescence antibody
MRC-5	human embryonic lung
NK2	monoclonal antibody to Namalwa IFN-α
NPS	nasopharyngeal secretion
PBS	phosphate-buffered saline

RHSC	Royal Hospital for Sick Children
rIFN-α	recombinant alpha-interferon
RNA	ribonucleic acid
RSV	respiratory syncytial virus
SGH	Southern General Hospital
SIDS	sudden infant death syndrome
URTI	upper respiratory tract infection
VTM	virus transport medium
Yok 5/19	monoclonal antibody to IFN-α
uCi	microcuries

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CHAPTER I

INTRODUCTION

Part I

Acute respiratory infections are the commonest illnesses in childhood comprising approximately 50% of all illnesses in children under five years (Phelan et al., 1982(a)). Among these infections, acute bronchiolitis is regarded as the most important respiratory illness in infants under six months of age, with a peak incidence at two to four months (Simpson et al., 1978).

It is now well established that respiratory syncytial virus (RSV) is the major cause of more serious respiratory infection in infancy. It is responsible for approximately 80% of cases of bronchiolitis, 12% of cases of laryngotracheobronchitis, 20% of cases of pneumonia and 15% of cases of bronchitis, in the first year of life (Phelan et al., 1982(a)). Although it appears to play a major role in the aetiology of bronchiolitis, the clinical features have also been described during infections with adenovirus types 3, 7 and 21, parainfluenza virus type 3, rhinovirus, herpes simplex type 1, certain serotypes of enterovirus and Mycoplasma pneumoniae (Simpson et al., 1978; Tercier, 1983).

1.1 EPIDEMIOLOGY OF RSV INFECTIONS

RSV presents a predictable pattern of illness

in the community. Epidemics have been reported from many countries, including Australia (Lewis et al., 1961), U.K. (Peacock et al., 1961; Mufson et al., 1973) where the peak of the epidemic corresponds with the coldest month of the year. In the United States there seems more seasonable variation in that the peak varies from late autumn, winter or early spring and usually lasts for 4-5 months (Martin et al., 1978; De Silva et al., 1981).

Host Factors

There are some predisposing risk factors which can cause a more severe disease. The pattern of illness varies considerably with age and with the genetic constitution of the infected person (Phelan et al., 1982(a)).

Sex

Although the sex of the patient does not affect the frequency of isolation of RSV, significantly more males than females are admitted to hospital with RSV infection in the ratio 1.3 or 1.4 to 1.0 (Stott et al., 1985).

Gestational age

During the first year of life infants born weighing less than 1500 g have a hospitalization rate due to RSV five times greater than infants born weighing more than 2500 g (Phelan et al., 1982(a)).

Congenital malformations

The risk of infection in babies with congenital malformations, especially cardiac disease who are admitted to hospital with RSV infection or who acquire it during hospitalization, have an increased risk of severe or fatal disease, mainly due to pulmonary hypertension. Infants with pulmonary hypertension are less able to compensate for the altered distribution of ventilation that occurs with RSV infection (McDonald et al., 1982).

Breast feeding

Observations of RSV infection related to breast-feeding halves the risk of admission to hospital in infants with RSV infection (Pullan et al., 1980). Many other social factors such as maternal care, a mother's smoking, and the presence of another child in the same room at night tend to be combined factors increasing the risk of infection (Phelan et al., 1982(a)).

Environmental factors

In a low income setting, the quality of maternal care seems to be the most important environmental factor in determining the likelihood of admission of an infant to hospital with RSV infection (Phelan et al., 1982(a)). Here, the family size is often larger, living quarters more crowded and children are more likely to be exposed to a relatively larger

infectious dose (Glezen et al., 1977; Glezen et al., 1981).

1.1.2 CLINICAL PICTURE OF RSV-BRONCHIOLITIS

The clinical picture of RSV-bronchiolitis is well known. The incubation period from exposure to first symptoms is about 4 days. The virus is excreted for variable periods, probably depending on severity of illness and immunological status (McIntosh, 1983).

Most infants with lower respiratory tract illness shed virus for 5 to 12 days after hospital admission and the first signs of infection of the infant with RSV are coryzal onset of one to several days followed by tachypnoea and signs of respiratory distress, sometimes accompanied by poor feeding (Simpson et al., 1978; McIntosh et al., 1980).

The chest becomes overinflated and a dry repetitive cough, restlessness and cyanosis may be present (Simpson et al., 1978). This hyperinflation of the lungs displaces the liver and spleen downward, so that they become easily palpable. These signs in an infant with bronchiolitis do not necessarily indicate heart failure (Reynolds, 1972). A cough appears more often after an interval of 1 to 3 days and the child begins to wheeze audibly. Auscultation often reveals diffuse rhonchi, fine-rales and wheezes (McIntosh, 1983).

The temperature is seldom above 38°C. Some infants with RSV bronchiolitis show signs of dehydration due to inadequate fluid intake and increase water loss from the respiratory tract (Simpson et al., 1978).

The white cell count is usually normal but when elevated the differential count may be normal or shifted either to the right or left (Reynolds et al., 1972).

Arterial blood gas estimations often show a low pO_2 and a normal or elevated pCO_2. The degree of elevation corresponds with the severity of the illness (Wohl et al., 1978).

The chest-X-ray findings are frequently normal. If the illness progresses, cough and wheezing increase, air hunger and evidence of hyperexpansion of the chest (particular intercostal and subcostal retraction) are evident (McIntosh, 1983).

The lung fields appear abnormally translucent and there are sometimes small areas of collapse (Reynolds et al., 1972). Peribronchial thickening or interstitial pneumonia is seen in 50 to 80 per cent of the cases and segmental consolidation occurs in 10 to 25% (McIntosh, 1983).

1.1.3 PATHOLOGICAL FEATURES

The earliest lesions occur in small airways ranging from 75-300 um in diameter. Histologically,

the most important lesion is obstruction of the bronchioles caused by thickening of the bronchiolar walls with oedema, lymphocytic cellular infiltrate and also by obstruction of the lumen with plugs of mucus and cellular debris (Simpson et al., 1978; Reynolds, 1972). In severe cases desquamation of the epithelial lining may occur. These changes are distributed throughout the lungs and the bronchi are much less severely affected than the bronchioles (Simpson et al., 1978).

1.1.4 **DIAGNOSIS**

The method for detecting RSV is by collecting respiratory secretions from the nasopharynx by aspiration using a mucus trap (Fenner et al., 1976). Because of the extreme lability of RSV, specimens are transferred straight into the culture tube at the bedside or transported at $4^{o}C$ to the laboratory for isolation and propagation (Treuhaft et al., 1985). Isolation from stored specimens is rarely successful but under favourable conditions HEp-2 (continuous human epithelioma cells) or (Bristol) HeLa cells (cervical carcinoma) are the most useful tissue culture cells for syncytial cytopathology (Pringle, 1985).

Human RSV can grow in a wide range of non-human cells and the relative susceptibility of different cells depends to some extent on the passage history of the virus strain employed (Pringle, 1985).

Thus, success in culturing the virus depends on frequent monitoring of cell lines for sensitivity (McIntosh, 1983).

Eagle's minimum essential medium (MEM), preferably supplemented with non-essential amino acids and glutamine is a satisfactory maintenance medium for RSV-infected cells (Pringle, 1985; Marquez et al., 1967).

Direct examination of RSV antigen can be detected in respiratory tract cells and in cells from virus isolation tubes by using the fluorescent antibody technique. It is an essential requisite for an unequivocal diagnosis by direct fluorescence that preparations contain a large number of intact nasopharyngeal cells (McQuillin et al., 1968). Monoclonal antibodies against RSV are preferable to animal anti RSV sera for use in antigen detection tests, since they have excellent sensitivity and specificity (Bell et al., 1983).

The use of monoclonal antibodies gives clear staining of cytoplasmic inclusions with very little non-specific fluorescence (Pothier et al., 1985).

The capability to provide a rapid diagnosis of RSV infection is an important clinical commitment, because of the need to control the potential spread of this infection in a hospital setting (Cheeseman et al., 1986).

Examination of acute and convalescent sera for a rise in complement fixation (CF) or neutralizing antibody to RSV is often disappointing in the infant. Nevertheless, if such a rise is found, the diagnosis is clear (McIntosh, 1983). In the convalescent period, a serological diagnosis by finding a high titre by CF or the presence of specific IgM, may be the only way of making a retrospective diagnosis.

1.1.5 **TREATMENT**

The management of the baby with RSV bronchiolitis depends on humidified oxygen therapy and good nursing care (Phelan et al., 1982(b)).

Severely affected babies are best nursed in a plastic oxygen cot which allows high concentrations of oxygen (Wohl et al., 1978). The hypoxaemia present in most infants is usually caused by ventilation-perfusion abnormalities and it is generally corrected with inspired O_2 of 30 to 40% (Wohl et al., 1978).

Intravenous or oral rehydration

Mild dehydration is common and in some infants fluids may have to be given for a day or two either by a gastric tube or by the intravenous route (Reynolds, 1972).

Antibiotic cover

As a virus disease antibiotics have no effect on the progress of the illness. At the first sign of secondary bacterial infection, a broad-spectrum

antibiotic has been known to be beneficial (Reynolds, 1972).

Bronchodilator drugs such as epinephrine, isoproterenol and aminophylline are commonly used in bronchiolitis, but they do not have a profound effect on the course of the disease (Reynolds, 1972).

Specific antivirals

Ribavirin is an antiviral drug that has recently been approved by the Food Drug Administration (USA) and the Committee on Safety of Medicines (UK) in an aerolized form for the therapy of RSV infection in those patients who do not require assisted ventilation. It is a synthetic nucleoside analog (1-β-D-ribofuranosyl-1,2,4,-triazole-3-carboxamide) resembling guanosine and ionosine; it appears to interfere with the expression of messenger RNA and inhibit viral protein synthesis.

Trials of ribavirin treatment of infants hospitalized with respiratory syncytial virus disease were begun in 1981 and have involved both normal infants and those with underlying diseases (Isaacs, 1987). It has been administered orally, intravenously and by aerosol to adults but only in aerosol form to children. Particles small enough to reach the lower respiratory tract (5 um) are delivered via an oxygen hood or tent for an average of three to five days for

18 hours each day.

Ribavirin aerosol was administered for only 12 hours in one study and the infants improved more rapidly than those given placebo, but the difference between the treated and placebo groups were less obvious than in another two trials that had a large dose (Taber et al., 1983). In these studies the treatment was administered for 18-20 hours each day and the difference in rate of recovery was most obvious for lower respiratory tract signs such as cough, tachypnoea, chest recession and crepitations in the lung fields (Barry et al., 1986; Hall et al., 1983).

Ribavirin may well have a place in the treatment of RSV bronchiolitis in infants who are likely to be severely affected, such as those with immunodeficiency, congenital heart disease, and bronchopulmonary dysplasia (Barry et al., 1986). At the present time, it has not been possible to anticipate the severity of the bronchiolitis during the course of the disease by observed parameters at the time of admission to hospital. This provides difficulty in deciding which cases should be treated with this costly preparation.

1.1.6 **PROGNOSIS**

The mortality of hospitalized infants with RSV infection of the lower respiratory tract is about 2

per cent (Simpson et al., 1978). The progress is clearly worst in infants with underlying disease of the neuromuscular, pulmonary, cardiovascular, or immunologic systems (Gardner et al., 1967; Downham et al., 1975; McIntosh, 1983).

A number of investigators have linked RSV infection with the sudden infant death syndrome (SIDS), because it has been described that a good proportion of infants with RSV lower respiratory tract infection die suddenly and unexpectedly. To date no studies have demonstrated evidence of a systemic viral infection indicated by viremia or elevated interferon (IFN) (Kelly et al., 1982). This is consistent with the findings in most studies involving viral isolation in SIDS where recovery of viruses has been limited to the respiratory and gastrointestinal tracts (Seto et al., 1978). One study has reported an association between infection with RSV and prolonged apnoeic episodes. In a small proportion of affected infants this progresses to the point where cardiac arrythmias followed by death occur with a fatal outcome (Bruhn et al., 1977).

Children who suffer acute bronchiolitis in infancy have a high incidence of respiratory problems in succeeding years, whether or not their initial illness was severe enough to require admission to hospital (Webb et al., 1985). More than 50% of children continue to wheeze recurrently. Atopy does

not seem to be an additional factor or play an important role since the low level of skin sensitivity has shown no difference between children with and without continuing symptoms (Sims et al., 1981; Webb et al., 1985).

1.1.7 **PREVENTION**

The efforts to develop an effective vaccine have been unsuccessful (Wohl et al., 1978). Approximately 20 years ago a formalin-inactivated RSV vaccine was evaluated in infants and young children. This vaccine stimulated moderately high levels of serum antibodies, as measured by the CF assay, but failed to induce resistance to infection caused by RSV (Kapikian et al., 1969). Infants receiving formalin-inactivated RSV vaccine developed more serious lower respiratory tract disease when infected with the wild virus at a latter natural challenge (Chanock et al., 1970). The treatment of RSV with formalin appears to have altered the epitopes of the F and G glycoproteins that stimulate neutralizing antibodies, with the result that the immune response consisted largely of non-functional antibodies. These may have participated in a pulmonary Arthus reaction during RSV infection leading to the increase in clinical sequelae (Murphy et al., 1986).

Vaccines prepared from temperature sensitive mutants of RSV have also proved unsatisfactory as the mutants used were unstable showing some latter reversion to wild type virulence (Chanock et al., 1977).

1.1.8 MECHANISMS OF THE DISEASE

Anatomical considerations

As adults complain mainly of upper respiratory tract symptoms, replication of the virus may be limited primarily to the upper airways (Wohl et al., 1978). The lack of severe symptoms referable to the small airways may therefore be related to an anatomic difference between the infant and the adult lung (Wohl et al., 1978). The conducting airways consist of 18 branching generations from the hilum and this series of dichotomous branches is completed by the sixteenth week of fetal life. Thus, at birth, the number of conducting airways is the same as in the adult lung. However, peripheral airways are disproportionately narrow in the early years of life (Hogg et al., 1968) and the disease in small airways would have a greater effect on increasing the resistance to flow in the young infant than in the adult (Wohl et al., 1978).

Immunological considerations

The available data on the possible immunological explanation for the severity of the

illness in the young infant are contradictory and difficult to interpret. Antibodies against some components of RSV may not only fail to protect but may be involved in the pathogenesis of severe bronchiolitis (Ogilvie et al., 1981).

Chanock and co-workers have postulated that the incidence of bronchiolitis is highest in the first 2 months of life, when maternally acquired antibody concentrations are greatest leading to a type 3 antigen-antibody response or Arthus reaction. He proposed that the interaction of serum antibody and viral antigens in the lungs plays an important role in producing the disease manifestations which are characteristic of RSV illness of early infancy, especially the bronchiolar obstruction of bronchiolitis (Chanock et al., 1970).

However, Lamprech and co-workers postulated that neutralizing antibody might be protective. They suggested that the presence of maternally acquired neutralizing antibody prevents spread of the disease (Lamprech et al., 1976). In a prospective study which studied one hundred newborn infants during a one year period, the authors suggest that the mean titre of maternal IgG antibody to RSV was significantly higher in those mothers whose babies remained uninfected than in those whose babies had proven RSV infection before six months of age (Ogilvie et al., 1981).

Other investigators have suggested that secretory IgA containing RSV antibody might be transmitted to the newborn in colostrum and breast-milk (Ogilvie et al., 1981; Downham et al., 1976).

A relationship between a low frequency of breast-feeding and a high incidence of RSV disease in infants was first suggested by Glezen and Denny (1973) in a survey of respiratory disease in the United States (Glezen et al., 1973). Further epidemiological evidence of the protective effect of breast-feeding demonstrated that a significantly lower percentage of infants admitted to hospital with RSV infection had been breast fed compared with age-matched controls (Downham et al., 1976).

The role of secretory IgA in protecting the infant has been studied by Scott and Gardner, who found IgA immunoglobulin to be consistently present at detectable concentrations. Neutralizing activity against RSV in secretions was due to specific IgA antibody (Scott et al., 1974).

Gardner and McQuillin have postulated that there is a prior sensitizing to RSV infection, leading to the production of IgE antibody. They suggested that the reaction is more akin to type 1 hypersensitivity. They found scant virus in impression smears of the lungs using immunofluorescence

staining in two children who died of bronchiolitis and abundant virus in one who died of pneumonia. Immunoglobulins (class unspecified) were present in the tissue preparation of bronchiolitis but not in those from cases of RSV-pneumonia. These observations were not consistent with a type 3 reaction which had been suggested by Chanock (Gardner et al., 1970(b); McIntosh et al., 1980).

Studies have demonstrated RSV specific IgE bound to exfoliated nasopharyngeal epithelial cells and histamine release from patients with all forms of illness due to RSV infection (Welliver et al., 1981). It appears to be more persistently present and in higher titre in patients with wheezing due to RSV infection than in patients infected with RSV without wheeze (Welliver et al., 1983(a)). The degree of RSV specific IgE responsiveness was highly correlated with the severity of illness as determined by the degree of hypoxia. It is postulated that, the production of RSV-specific IgE and release of histamine may determine the form and severity of illness in most cases of lower-respiratory tract illness due to RSV (Welliver et al., 1981).

The relationship of suppressor cell numbers and function to virus-specific IgE response was determined in children with RSV infection (Welliver et al., 1984). Patients with bronchiolitis had fewer

OKT8-positive cells during convalescence than patients with other forms of illness due to RSV and histamine-induced suppression was also reduced in patients with bronchiolitis (Welliver et al., 1984).

1.1.9 IMMUNOLOGICAL ASPECTS

It is not fully established if the neonate is immunocompetent with regard to his immunological functions, since there are numerous quantitative differences compared with the adults or older children (McIntosh, 1980). However, studies have shown that RSV specific secretory IgA as well as IgG and IgM, appear in the respiratory tract as early as the first three days after the onset of illness caused by RSV infection (Welliver et al., 1983(b). The capacity of lymphocytes of newborn to mediate antibody dependent cellular cytotoxicity against virus-infected target cells is thought to be normal (McIntosh, 1980). Lymphotoxin production by activated lymphocytes is reduced in cord blood but IFN production, on the other hand, appears to be normal (McIntosh, 1980; Ray et al., 1967).

Despite all the scientific work over the past 20 years to discover the pathogenesis of RSV-bronchiolitis, a final verdict on this has not been revealed.

Several theories of the pathogenesis of RSV disease have been proposed, based on the immunological

immaturity of the infant, a role for serum antibody, a delayed hypersensitivity-type reaction, an IgE-mediated reaction and non-immunological mechanisms (Stott et al., 1985). Clearly, no firm conclusions are possible about which immunopathologic mechanisms in bronchiolitis should be explored, but interferon studies have been few. As techniques have advanced in interferon detection the time is opportune to investigate this aspect further.

CHAPTER I

INTRODUCTION

Part II

1.2.1 CURRENT CONCEPTS OF INTERFERON

The discovery of interferon (IFN) by Isaacs and Lindenman in 1957 was a direct consequence of preceding studies on interference of the infectious agent in the allantoic cavity of the chick embryo (Isaacs et al., 1957; reviewed by Henle et al., 1984).

The initial papers suggested that IFN may have been a single substance produced by all cells in response to all viruses (reviewed by Burke, 1985). However, it was shown that IFN made in calf kidney cells was inactive in chick cells and it was inferred that IFN was only active in the tissue in which it was formed, demonstrating species-specificity (Tyrrell, 1959).

It is now known that IFN is a family of proteins and glycoproteins. They are divided into three major classes, initially determined by the cell type in which they are predominantly produced: leucocyte for IFN-α, fibroblast for IFN-β and lymphocytes for IFN-γ (Zisman et al., 1985).

In 1983 a more rational nomenclature was introduced, since it was found that agents other than viruses could induce IFN. A distinction was therefore made between Type 1 (induced by viruses and double-

stranded RNAs) and Type II (induced by mitogens) (Burke, 1985). There are at least 20 types of IFN-α 2 subtypes of IFN-β, and a single subtype of IFN-γ (Knight, 1984).

1.2.2 IN VITRO ASPECTS

IFN is well established as a natural consequence of viral infection, but the degree of IFN induction varies between agents. The basic requirements for induction is an effective interaction between the virus particle and the cell (Ho, 1984). There are a number of factors which can affect the ability of viruses to induce IFN, such as metabolic effects of viruses on the cells, addition of hormones, carcinogens and ultra-violet light (Ho, 1966).

Besides animal viruses, a large number of microbial agents, including viruses from fungi, bacteria and plants, and unicellular organisms can induce IFN (Ho, 1984).

Despite their wide variety, all IFN-inducing microorganisms appear to be cell associated parasites. It is possible that they have a common feature in their host cell relationship which is critical for IFN production (De Clercq et al., 1970).

Some gram-negative bacteria such as Brucella, Salmonella, Serratia, Bordetella, Francisella and Hemophilus and few gram-positive bacteria such as

Listeria, Staphylococcus and Corynebacterium have been reported as IFN inducers (Ho, 1984).

1.2.3 In VIVO ASPECTS

Using biological and immunological assays it has been possible to determine the biological activity and potency of the particular IFN which is under investigation (Grossberg et al., 1984). The choice of a particular IFN assay depends on several factors such as the number of samples to be assayed, the range of levels of activity, the need for precision and sensitivity, rapidity, ease of performance, economy and each investigator must decide which type of assay best suits his own circumstances (McNeill, 1981).

1.2.4 BIOLOGICAL ASSAYS

The biological assays are based on the ability of a preparation to inhibit the production of a virus or a viral product (Friedman et al., 1983).

Biological assays are laborious and less precise in comparison with most immunological assay procedures. They all depend on a biological property, the ability of IFN to inhibit the growth of viruses (Finter, 1973). The characteristics of an assay method are therefore determined by the particular way it is used to measure virus growth. Different ways have been used in different IFN assays, and some of the resulting assays have proved to be much more satisfactory than others (Finter, 1973). In a typical

antiviral bioassay a series of dilutions of the IFN are made and each is added to one or more replicate tissue cultures of an appropriate cell (Finter, 1981). The cultures are incubated usually overnight and in those with a sufficient amount of IFN, an antiviral state develops in the cells (Finter, 1981). The cultures are then all challenged with a convenient virus e.g. Semiliki forest virus, Sindbis virus etc. After further incubation for an appropriate time, the extent of virus growth in each culture is determined in some way. The relation between the amount of virus growth and the amount of IFN is established graphically by calculation (Finter, 1981).

In order to be able to carry out an IFN assay, it is essential to have some basic familiarity with tissue culture methods, sterile handling techniques, and the virus concerned. Moreover, from a sensitivity point of view, as well as convenience, continuous lines are preferred as the substrate for IFN assay (Finter, 1981). It is advisable to obtain fresh supplies of these cells at regular intervals of 2-3 months in order to avoid inapparent contamination with mycoplasma or latent viruses which can alter assay results (Grossberg et al., 1984).

The viruses used for IFN assays should be laboratory adapted strains of relatively harmless agents (Finter, 1981). The quality of animal sera

used in cultures and water employed in medium should be carefully controlled (Grossberg et al., 1984). Clearly bioassays are extremely time consuming and technically demanding and suitable only for research laboratories.

The most popular assays for IFN are those which depend upon using a virus which causes extensive damage to the cultured cells so the potency of an IFN preparation is then determined by its ability to prevent virus infection (Grossberg et al., 1984).

The plaque-reduction method

The principle is that virus released from an infected cell in a monolayer is restricted to the vicinity of that cell because agar or some other viscous agent is included in the medium covering the cells. The released virus is able to infect neighbouring cells, so that clusters of virus-infected cells eventually surround each cell successfully infected by a virus particle in the inoculum. These clusters, which are termed plaques, can usually be counted with the naked eye, they are large enough but not so numerous that they fuse with one another, especially if the monolayers are stained with neutral red. Living cells take this up and are stained red, but cells killed as the result of virus growth lose their stain, and so the plaques are seen as pale areas on a red background (Finter, 1973).

Established cell lines commonly used to assay human IFN by this method are human amnionic cells (WISH), human foreskin fibroblast. Rabbit kidney and mouse L929 cells are used for rabbit and mouse IFN, respectively (Langford et al., 1981).

The most commonly used challenge virus for plaque reduction assays has been vesicular stomatitis virus (Langford et al., 1981; Grossberg et al., 1984). Variations in IFN titres with the plaque reduction assay is primarily dependent upon changes in sensitivity of the cells to IFN and virus.

To overcome some of the difficulties associated with doing plaque-reduction assays, microplaque-reduction assay for mouse and human IFN has been devised which gives titres comparable to those obtained with the usual plaque-reduction system (Grossberg et al., 1984).

Virus-yield reduction assay

In yield reduction assays, virus growth is measured directly as infectious virus, haemagglutinin, haemadsorbing protein, viral enzyme (neuraminidase) or viral nucleic acid (Oie, 1977). These assays generally use high multiplicity of challenge to infect all the cells at the outset and determine the extent of inhibition of the viral parameter after a single cycle of viral growth (Grossberg et al., 1984).

Reduction of infectious virus yield

This assay consists of three sequential parts. The first one is to incubate IFN dilutions with sensitive cell cultures. After that the challenge of IFN-treated cells with a high multiplicity virus and finally measurement of the degree of inhibition of viral yield (Weigent et al., 1981).

Routine assay of IFN by the yield-reduction method is not usually performed because it is expensive and slow and requires more time and effort than other IFN assays (Oie, 1977). However, this assay is the method of choice when it is required to investigate the temporal changes in antiviral activity that occur within a cell following IFN treatment and the ultimate effect of these changes on the virus growth cycle (Weigent et al., 1981).

The challenge virus most commonly used is Sindbis virus because it grows well in a variety of cell types used for IFN assays, it is very sensitive to IFN and forms plaques well in primary chick embryo cell and baby hamster kidney cultures (Weigent et al., 1981).

The disadvantages of Sindbis virus in yield reduction assays is that virus infectivity decreases with storage, freezing and thawing. Therefore, virus samples should be assayed directly or stored at -70^{o}C for a short period of time (Oie, 1977).

Haemagglutinin (HA) yield reduction

The reduction in yield of influenza virus haemagglutinin from infected chicken embryo chorioallantoic membrane fragments was used by Isaacs and Lindenmann as the first method of measuring IFN antiviral activity (Jameson et al, 1981).

Many different types of IFN assay methods have been developed subsequently in an effort to improve sensitivity, precision, speed and convenience of measurements (Jameson et al., 1981). The typical assay consists of four steps: first preparation of cell cultures, exposure of cells to IFN, infection of washed cell culture with virus and measurement of virus yield (Jameson et al., 1981). Recently, an HA yield-reduction method using encephalomyocarditis virus has been applied to measurements of IFN in man, monkey, cow, pig, rabbit, cat, hamster, dog and mouse cells (Grossberg et al., 1984; Jameson et al., 1981). HA yield-reduction assays are practical, sensitive, rapid, objective, reproducible and have been adapted to microtitre methods (Oie, 1977).

Neuraminidase yield-reduction

The reduction in yield of influenza A virus neuraminidase is a sensitive and reproducible bioassay for various species of IFN (Grossberg et al., 1984).

A recombinant virus X7(F1)(HON2) was the most IFN sensitive of 12 influenza viruses tested and also

produced higher yields of neuraminidase in most cell cultures than the other influenza viruses (Sedmak et al., 1981). This assay is based on the reduction of neuraminidase during a single cycle of influenza virus in chicken embryo cell cultures (Oie, 1977). This method is rapid, convenient, reproducible and is as sensitive as other methods (Oie, 1977).

Inhibition of viral RNA synthesis

The assay of IFN by measuring its inhibitory effect on viral RNA synthesis was developed and found to be rapid, reproducible and highly sensitive (Giron, 1981).

The assay measures the incorporation of (3H) uridine into MM virus-infected cells in the presence of actinomycin D, which inhibits DNA-dependent RNA synthesis but allows picornavirus RNA synthesis to proceed (Grossberg et al., 1984).

Cytopathic effect (CPE) inhibition assay for interferon

These assays depend upon using a virus that causes extensive damage to the cultured cells. The potency of an IFN preparation is determined by its ability to prevent virus-induced CPE (Grossberg et al., 1984).

Cell cultures are prepared and when monolayers have formed are treated with each of a series of IFN dilutions. After a suitable incubation period to permit establishment of the antiviral state,

the IFN is decanted and the cultures are exposed to an IFN-sensitive challenge virus and incubated for 24-48 hours to permit development of extensive cytopathology in unprotected cultures (Armstrong, 1981). The medium is then decanted, the cultures are fixed and stained with crystal violet and dried in air. Solvent is then added to each well to elute the dye, and the topical density of the eluent is measured to give a measure of the degree of cytopathology in cultures exposed to dilutions of IFN (Armstrong, 1981).

For human IFN, various diploid human fibroblast may be used, including those of foreskin and embryonic skin/muscle origin and for human, rabbit and mouse IFN assay vesicular stomatitis virus is used as a challenge virus. The most important point in this type of assay is to choose a virus which causes a marked and characteristic CPE in the particular test cell (Finter, 1973). The assay is quite economical, entirely objective and quantitative.

Dye uptake method for assay interferon activity

The dye uptake method, a modification of the CPE inhibition assay, indirectly measures the extent of CPE damage to cells by the amount of vital dye, neutral red, taken up by cells. Cell taking up the dye can be microscopically enumerated, or cell-bound dye could be extracted and colorimetrically estimated (Oie, 1977).

In contrast to the CPE inhibition method, the dye uptake assay is objective in addition to being reproducible, sensitive and economical (Oie, 1977). Cells commonly used are vervet monkey kidney line, V3, but Vero cells are also satisfactory and Semiliki Forest virus is used as a challenge (Johnston et al., 1981).

1.2.5 **IMMUNOASSAYS**

In view of the lack of sensitivity of most biological assays to measure IFN below 10 IU/ml, immunoassays were recently introduced. Radioimmunoassays by several different methods has been the most versatile and common method used.

Secher and Burke (1980) have produced a monoclonal antibody specific for human interferon alpha (HuIFN-α) to Namalva IFN (NK2) and Secher has shown its usefulness for an immunoradiometric assay (IRMA). The antibody was used to purify HuIFN-α by immuno-adsorption chromatography and after labelling with ^{125}I, immunoradiometric assay was developed (Secher, 1981).

This assay uses a crude sheep anti HuIFN-α antibody of known potency bound to a solid phase (sepharose) to which an IFN preparation is added. The amount of IFN bound to the solid phase could then be calculated from the amount of binding of radiolabelled monoclonal antibody (Grossberg et al., 1984).

IRMA offers considerable advantages over the conventional biological assays. Since the antibody-coated sepharose can be stored at $4^{o}C$, samples can be assayed at short notice and the results are obtained within 24 hours of beginning the assay (Secher, 1981). The assay is semi-automated and hundreds of samples can be assayed together at one time (Secher, 1981). This assay can measure concentrations of 50 IU/ml of IFN and it has a standard error of 10% for independent assays on the same sample (Grossberg et al., 1984).

A modification of this assay was performed by Walker and co-workers for the measurement of low concentrations of HuIFN-α in serum. The sensitivity of the assay is 5 to 10 IU/ml and the coefficient of variation less than 10% (Walker et al., 1982).

Moreover, a rapid solid phase sandwich immunoassay for the detection and quantitative determination of HuIFN-α was described. Two monoclonal antibodies were selected which recognize a different epitope of human leucocyte IFN and they bind to IFN simultaneously without mutual interference (Staehelin et al., 1981). One monoclonal antibody is immobilized on a solid surface to which the IFN dilutions are added. The second one, labelled with ^{125}I or an enzyme, binds proportionately to the amount of IFN bound to the first antibody. After incubation

and washing, IFN concentration is determined by measuring the amount of labelled antibody. This radioimmunoassay has already proven extremely useful for monitoring IFN during purification and the sensitivity approaches that of the CPE inhibition test (Staehelin et al., 1981).

The other type of radioimmunoassay reported for IFN is a competitive inhibition assay for HuIFN-α which use highly purified ^{125}I-labelled HuIFN-α, rabbit anti-HuIFN-α IgG and protein A-containing as an IgG reagent (Daubas et al., 1982).

The sample to be assayed is incubated with purified rabbit anti-HuIFN-α immunoglobulin and the highly purified ^{125}I HuIFN-α is added. The mixture is incubated overnight after which Staphylococcus aureus containing protein A is added. The immune complexes bound to the S.aureus are washed, centrifuged; and the amount of label in the complex is determined (Grossberg et al., 1984). The lower limit of sensitivity of this assay is 10 IU/ml. As the antibody is not monospecific for IFN, the specificity of the assay depends on the purity of the labelled IFN. The technique may offer a greater probability for recognizing more HuIFN-α species than do assays using monoclonal antibody (Daubas et al., 1982).

Mouse monoclonal antibody directed against HuIFN-α was coupled to sepharose and used as an immunoadsorbent to purify HuIFN-α. In addition, the IFN preparation was iodinated for use as a probe in a radioimmunoassay for IFN-α (Meurs et al., 1982).

Another IRMA using a ^{125}I labelled rat monoclonal antibody (designated Yok 5/19) to leucocyte IFN-α has been developed by Boots Celltech Diagnostics Limited. The assay has two site IFN capture, with a solid phase (sepharose) coupled to sheep anti-IFN. The assay can detect as little as 0.5 IU/ml of IFN-α in serum, this detection limit being the IFN-α concentration that gives a radioactive signal at zero IFN-α, and shows negligible cross-reactivity with HuIFN-β (<0.015%) and HuIFN-γ (<0.005%). The intra-assay co-efficient of variation was typically <10% over the range 3.55-7.30 IU/ml IFN-α during the first 8 weeks of products shelf life (Abbott et al., 1984). A radioimmunoassay has also been developed for HuIFN-β.

This immunoassay consists of incubating HuIFN-β with rabbit anti-HuIFN-β immunoglobulins immobilized on sepharose, and incubating the resulting solid phase IFN antibody complex with purified polyclonal ^{125}I-labelled anti HuIFN-β immunoglobulins. The amount of ^{125}I labelled anti HuIFN-β bound to the complex was linearly proportional to the amount of antiviral activity present in the complex (Inoue et

al., 1981). This assay can detect as little as 5 IU/ml and the entire assay is completed in 5 hours (Grossberg et al., 1984).

At present monoclonal antibodies to HuIFN-β suitable for immunoassays have not been developed.

Kelder and co-workers developed a technique for the detection and assay of HuIFN-γ. The principle of the assay is identical to that described by Staehelin. Since a pair of monoclonal antibodies are used in this assay, only those IFN-γ molecules that are recognized by both monoclonal antibodies are detected. This radioimmunoassay could be used for monitoring IFN-γ during purification or for surveillance of IFN-γ levels in patients for pharmacokinetics studies (Kelder et al., 1986).

1.2.6 IN VIVO ASPECTS OF INTERFERON REPORTS DURING INFECTIOUS DISEASE

IFN has been presumed to play a major role in recovery from viral respiratory infection, especially in the primary infection with a specific respiratory virus. The peak of IFN responses occur early in the course of infection before detectable antibody and is probably responsible for the early reduction of the virus shedding (Moehring et al., 1971).

A study conducted in different sets of mice showed that in the primary infection with influenza aerosols, the peak levels of IFN in lungs lavages and

the rapid production of antibodies in serum of mice were reached before detection of significant levels of neutralizing antibodies in serum and lung lavage (Iwasaki et al., 1977; Zee et al., 1979).

Higher IFN levels were found in sets of mice with higher mortality rate and it is apparent that the production of IFN is a direct consequence of virus replication in the lung tissues of infected animals (Zee et al., 1979).

High IFN titres were detected in the early stage of infection in calves after bovine RSV infection which dropped to undetectable amounts for at least one week and reappeared in low amounts and persisted for a number of weeks (El Azhary et al., 1981).

Deficient production of IFN-α has been described and has been associated with undue susceptibility to respiratory viruses (Isaacs et al., 1981). The inability to produce IFN was found in children with prolonged or recurrent respiratory infections and in those treated with prednisone and it could explain the negative influence of cortico-steroid therapy on the course of acute viral infections (Vanecek et al., 1985).

Wheelock and Sibley in 1964 investigated IFN responses by bioassay in human serum during clinical viral infections (Wheelock et al., 1964). Later, Matthews and Lawrence, measuring IFN levels in body

fluids as a rapid test, were able to demonstrate coincident production of IFN during acute viral infection (Matthews et al., 1979). Subsequently, in a series of serum studies, it was found that IFN determinations might be useful in establishing a viral aetiology (Parry et al., 1981; Levin et al., 1981; Flowers et al., 1985).

Significantly elevated IFN titres were found in sera and cerebrospinal fluids (CSF) of patients suspected of having viral infection, as compared to healthy controls. Moreover, these investigators suggest that detection of IFN in these fluids may be useful in suspected herpes encephalitis, where antiviral drugs should be administered as soon as possible (Negreanu et al., 1983).

Using a IRMA based on Yok 5/19 monoclonal it was possible to detect biological levels in vivo of 1.5 to 100 IU/ml in CSF of patients with suspected meningitis (Ho-Yen et al., 1987). IFN-α levels were found to be useful in distinguishing between bacterial and viral meningitis and were a better guide to viral meningitis than differential CSF cell count.

Local and systemic IFN production has been reported in children with a variety of acute viral respiratory infection (Ray et al., 1967; McIntosh et al., 1978) mainly in children infected with parainfluenza or influenza viruses, whereas only a few

TABLE 1

Interferon titres found in sera, CSF and NPS of patients with different viral infections

Study	Viral causes	Diagnosis	Levels of interferon	Specimen	Interferon test
Ho-Yen et al., 1987	Echo virus type 7,9,11,14 Coxsackie A9,B5 Mumps	Meningitis	1.5-100 IU/ml	CSF	IRMA
McIntosh, 1978	RSV Influenza	Virus infection	>4-16 IU/ml	NPS	Virus yield reduction
Breese et al., 1978	RSV Flu A Paraflu 1	Acute respiratory disease	5-10 IU/ml 5-86 IU/ml 5-90 IU/ml	NPS	Plaque reduction assay
Wheelock et al., 1964	No virus isolation	Viral infections	10-500 IU/ml	Serum	Plaque reduction assay
Negreanu et al., 1983	Herpes Mumps Echo 9 Measles Adeno type 2	Suspected viral infection	12-75 IU/ml $\geq$12 IU/ml/CSF $\geq$12 IU/ml/serum	Serum CSF	Dye-binding semi-microassay

children with RSV infection had detectable IFN titres in their nasal secretions (Hall et al., 1978; McIntosh, 1978) (Table 1).

As RSV is a member of the Paramyxovirus group, these observations are surprising since members of this group of viruses are normally very good inducers of IFN (Corbitt, 1971). The very low multiplicity obtainable in vivo, could explain the very low levels of IFN activity detected. These low levels may be inadequate to protect other cells in the respiratory tract from further RSV infections (Chonmaitree et al., 1981).

However, appearance of IFN in nasal secretions in a limited number of RSV infected patients did not correlate with cessation of viral shedding (Hall et al., 1978). Despite the evidence from other studies, it has been suggested that RSV is sensitive to the antiviral action of IFN (Corbitt, 1971; Gardner et al., 1970(a); Moehring et al., 1971).

The failure to detect IFN-α in the majority of nasopharyngeal secretions (NPS) during RSV infection, may reflect the apparent natural lack of induction or a limitation of the method used. As biological methods lack sensitivity, a re-evaluation of IFN positivity of RSV in nasopharyngeal secretions (NPS) with more sensitive techniques may provide future useful information in the pathogenesis of RSV-

bronchiolitis (Chonmaitree et al., 1981).

This study attempts to determine the usefulness of a sensitive solid-phase IRMA for IFN-α in the context of an acute RSV infection to determine whether IFN positivity can be correlated with the severity of disease and provide a helpful indicator to physicians with respect to management or treatment of this disease during the course of the illness.

CHAPTER II

2.1 PATIENTS, MATERIALS AND METHODS

2.1.1 Patients

The study group consisted of 208 children (128 males, 80 females) aged between 15 days to 6 years (mean: 8.8 months) admitted to the Royal Hospital for Sick Children, Glasgow, between October 1985 to March 1986.

Details of the geographical location of residence of each patient were collected to follow the epidemiological and social impact of RSV on the paediatric population of Glasgow. However, of the total number of children only 193 came from the Glasgow area and the remainder from other parts of Scotland. Considering the five districts of the Greater Glasgow Health Board, the majority of cases were from Glasgow City (137) followed by Clydebank (25 cases), Strathkelvin (13 cases), Eastwood (11 cases) and Bearsden/Milngavie (7 cases).

Patients were not excluded for prior antibiotic treatment, or selected with regard to the number of symptomatic days prior to the admission. A full history and clinical examination was carried out in each case, and data recorded on a special form

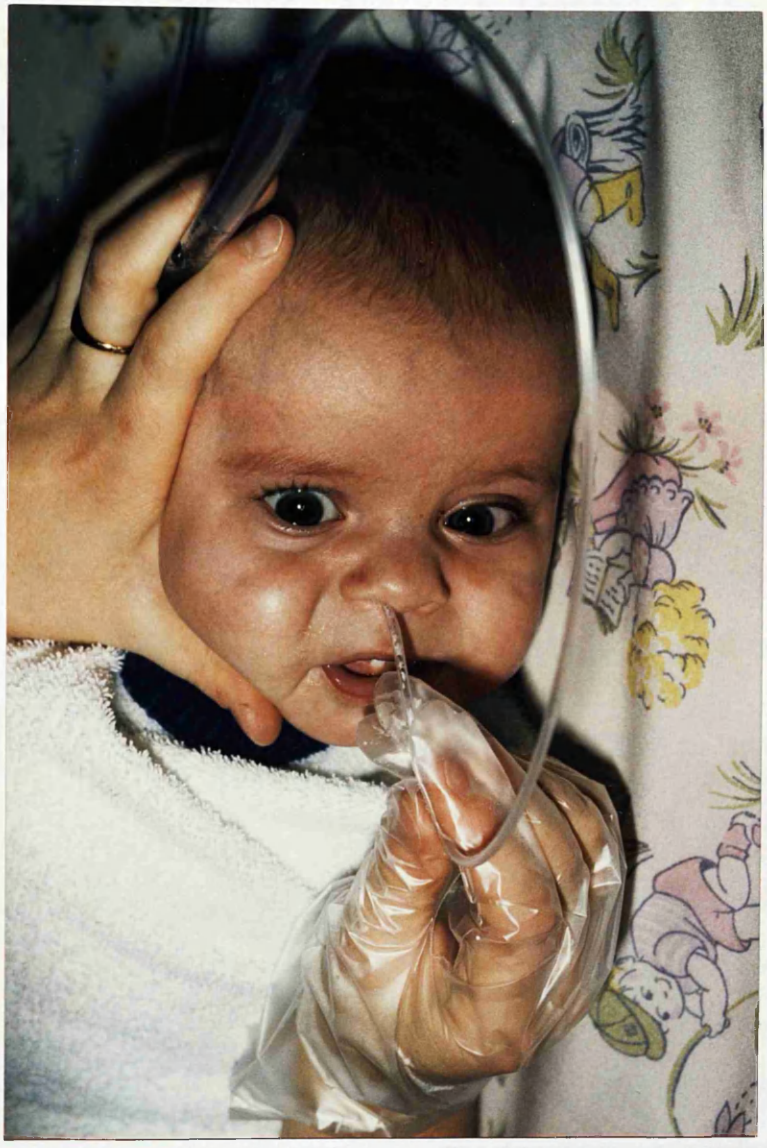


Figure 1:

Plastic disposable collecting mucus trap with an attached feeding tube to obtain secretions

suitable for computer analysis (Appendix 1). This included admission history, diagnosis, classified into upper respiratory tract infection (URTI), pneumonia, bronchiolitis, chronic lung disease and other.

Bronchiolitis was considered in three major groups: mild, moderate and severe. Laboratory findings of immunofluorescence technique, viral culture and IFN-α levels were also included.

For this study special consideration was given to clinical parameters such as maximum respiratory rate, days of physiotherapy, time to establish normal respiratory rate etc, which might indicate the degree of respiratory impairment or constitutional upset.

2.1.2 **Specimens**

The study was approved by the Research and Ethics Committee of the Royal Hospital for Sick Children, Glasgow. Nasopharyngeal secretions were collected on admission, or first day of symptoms if already admitted, and subsequently repeated on days 3, 5 and 7 when possible.

Plastic disposable collecting mucus traps with an attached feeding tube (5 fg) were used to obtain the secretions. The tube was passed into the posterior nasopharynx and suction at a vacuum pressure up to 25-50 mm of Hg was applied (Figure 1).

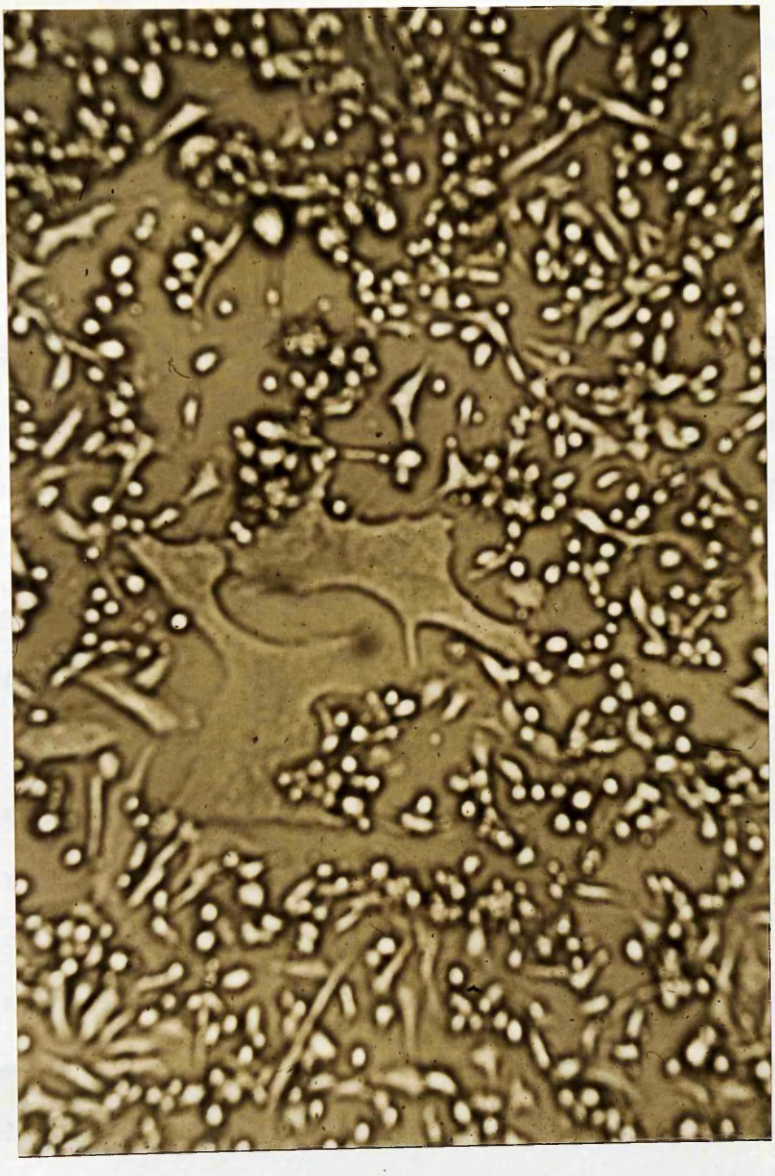


Figure 2:

Characteristic RSV-syncytial formation. Magnification x 100.

If only the tube contained secretions it was cut from this trap and it was sent in a universal container to the laboratory. A portion of the secretions was cultured in bacteriological media and the remainder was transferred to virus transport medium (VTM) in a 1:10 dilution. After centrifugation of the specimen suspension (1500 x g for 15 minutes), the supernatant fluid was used for virus isolation and assay for IFN-α. The cell pellet was examined by direct immunofluorescence using an RSV specific monoclonal antibody.

2.1.3 **Viral isolation**

RSV isolation

Duplicate tubes of HEp-2 were inoculated with 0.2 ml of specimen, incubated at 37^{o}C and observed daily for characteristic RSV-syncytial formation (Figure 2). After seven days of incubation, one tube was kept for passage and the other tube was used for detection of RSV using immunofluorescence. The monolayer was removed with trypsin/versene and resuspended in 0.1 ml of phosphate-buffered saline (PBS).

A capillary tube was used to place two drops on multiwell test slide. These were allowed to air dry and then fixed in acetone for 10 minutes. This slide was examined by direct immunofluorescence (see Section 2.1.4).

Other respiratory viruses

Cell cultures of rhesus monkey kidney and MRC-5 (human embryonic lung) were inoculated with 0.2 ml of the same specimen and were incubated at 37°C. Cultures were examined microscopically for typical CPE for 18 days before being discarded as negative. Adenoviruses were untyped but confirmed by electron microscopy examination of the tissue culture fluid.

2.1.4 RAPID DIAGNOSIS BY THE DIRECT IMMUNOFLUORESCENCE TECHNIQUE

RSV antigen was detected in exfoliated respiratory tract cells in cells from virus isolation tubes using a monoclonal antibody preparation "Imagen" commercially available (Boots Celltech Diagnostic Limited, Slough, U.K.) The Imagen reagent consists of a pool of purified murine monoclonal antibodies directed against the fusion and nucleoproteins of RSV; the antibodies are conjugated to fluorescein isothiocyanate and provided at a working dilution with Evans blue counterstain (Cheeseman et al, 1986). A capillary tube was used to place two drops on a multiwell test slide. These were allowed to air dry and then fixed in acetone for 10 minutes. After acetone fixation, 20 µl of fluorescein isothiocyanate (FITC) conjugated monoclonal antibodies which bind specifically to RSV was put on the appropriate wells which were incubated in a humidified chamber at 37°C

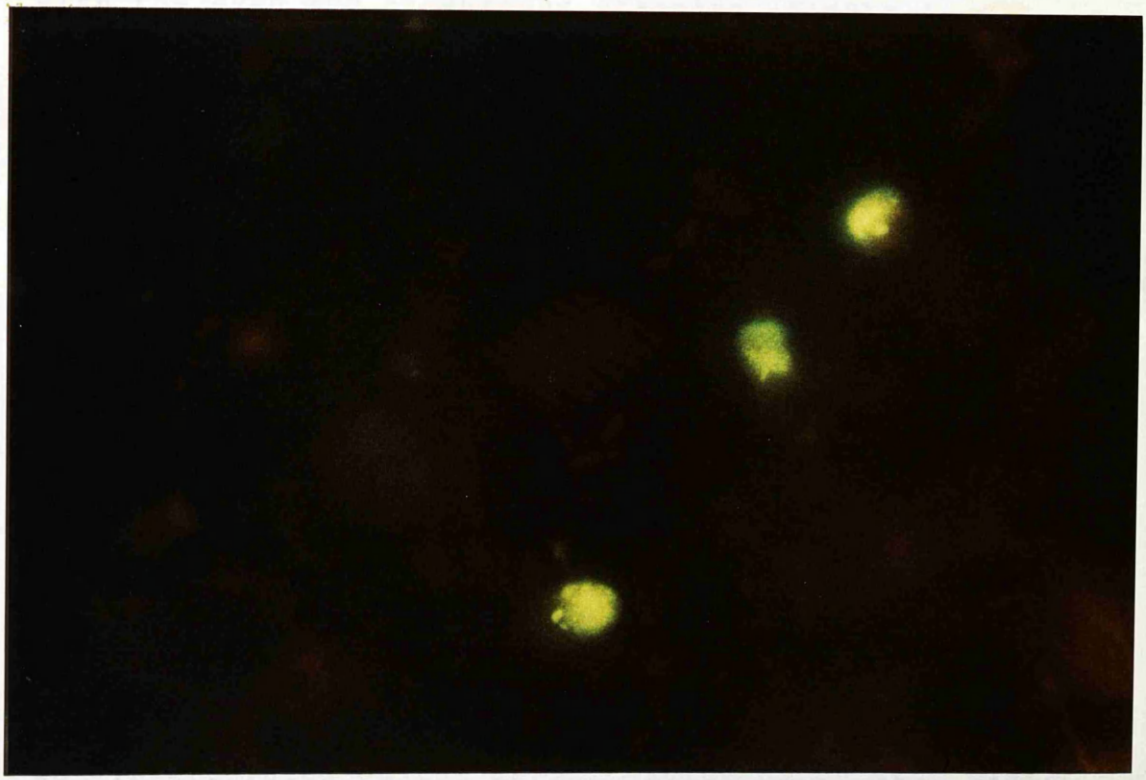


Figure 3:

Rapid diagnosis of RSV by direct monoclonal antibody immunofluorescence technique showing granular cytoplasmic fluorescence. Magnification x 600.

for 20 minutes. Slides were then washed once in PBS and distilled water for 5 minutes, allowed to dry and finally examined under a fluorescence microscope (Nikon Episcopic-Fluorescence attachment EF-D) at magnification of x 50 (oil). If RSV antigen was present, characteristic granular bright green contrast with the even red background staining (Figure 3).

2.1.5 **BACTERIOLOGICAL EXAMINATION OF NASOPHARYNGEAL SECRETIONS**

Nasopharyngeal secretions underwent bacteriological examination using the following procedures. Each specimen was plated onto five culture media: one horse blood agar and one chocolate agar were incubated in an atmosphere of 5-10% CO_2: one horse blood agar was incubated anaerobically: A MacConkey agar and a Sabouraud Dextrose agar were incubated aerobically. All plates were incubated overnight at 37°C. A single streak of Staph. aureus was incorporated on the aerobic horse blood agar plate perpendicular to the butt for the detection of satellitism as demonstrated by colonies of Haemophilus influenzae.

To aid presumptive diagnosis of group A Streptococci, a bacitracin (Oxoid Ltd, England) disc was placed onto the anaerobic horse blood agar plate. Beta-haemolytic Streptococci which failed to grow on MacConkey agar and which were inhibited by the

bacitracin in a zone at least 12 mm in diameter were considered to be Streptococcus pyogenes.

Other beta-haemolytic streptococci were identified using the Streptococcal Grouping Kit (Oxoid Ltd, England) or, if this proved inconclusive, the API STREP (API Ltd, Basingstoke, England) identification kit.

An optochin (ethyl hydrocupreine hydrochloride) (Oxoid Ltd, England) disc was placed on the horse blood agar plate, incubated in CO_2 for the presumptive identification of Streptococcus pneumoniae. Alpha-haemolytic colonies which showed a zone of inhibition around the disc at least 12 mm in diameter were identified as Streptococcus pneumoniae. Isolates of Streptococcus pneumoniae were serotyped using agglutinating antisera (Inverclyde Biologicals).

Any organism showing satellitism around the Staph streak was stained by Gram's method. Gram-negative bacilli were speciated by determining their requirement for X and V factors.

Coliform bacteria were identified using the API 20E identification kit (API Ltd, Basingstoke, England).

Yeast isolates were identified by the germ-tube test. Germ tube negative isolates were sent to the Medical Mycology Unit at Anderson College, University of Glasgow for speciation.

Where necessary, antibiotic sensitivity tests were carried out using a disc diffusion technique.

2.1.6 Embryonated hen egg inoculations

Freshly fertilised eggs from a local farm were incubated for 9-11 days in a humidified incubator and turned regularly. Before inoculation, the eggs were checked for infertility by candling.

Embryonated hens' eggs were inoculated in triplicate with NPS by the amniotic route. Initially eggs were candled to mark the air space. Shells were then cut away above the air space and the chorioallantoic membrane was cleared with paraffin to mark the position of the embryo.

With a 1 ml syringe fitted with a 1.5 inch, 22 gauge needle, the inoculum was injected into the amniotic cavity. The shell was sealed and the eggs were incubated for three days at 33°C.

After this period, eggs were placed in a 4°C refrigerator overnight to prevent bleeding from ruptured vessels during harvesting. The shell membrane was removed aseptically and amniotic fluid was harvested using a tuberculin syringe and 23 gauge needle. Haemagglutination tests were performed using twofold dilutions in 0.05 ml volumes of PBS in a microtitre plate. Fowl red cell suspension at 0.5% was added to each test well including an erythrocyte control well

which contained 0.05 ml of PBS.

Erythrocytes in the test wells were mixed and plates were incubated at 4°C for one hour or until control cells have settled.

Plates were inspected and the haemagglutination titre was defined as the reciprocal of the highest dilution of each sample causing complete agglutination when mixed with an equal volume of 0.5% red blood cells.

Chicken red blood cells, but not guinea pig red blood cells will be agglutinated by influenza C virus. Influenza C and some newly harvested influenza A viruses may elute rapidly from the erythrocytes at ambient temperatures, so the settling of virus-erythrocytes was performed in the cold.

CHAPTER III

3.1 INTERFERON ALPHA ASSAY PROCEDURE

IFN-α levels were determined by an IRMA "Sucrosep-IFN-α" commercially available from Boots Celltech Limited, Slough, U.K.)(Figure 4). This assay has been used primarily to study IFN-α levels in serum. In this study its use has been extended to determine levels in NPS.

Specimens were cleared as much as possible from cellular or other particulate material removed before analysis.

The test utilises an ^{125}I labelled highly specific monoclonal antibody (Yok 5/19) to leucocyte IFN-α. There are two sites of IFN capture. Any IFN-α present in the specimen is captured by radioactively labelled monoclonal antibody.

A second polyclonal linked to a high molecular weight particle is then added which binds labelled IFN-α present. The solid phase (sepharose) polyclonal antibody sediments to the base of the test tube, carrying with it bound labelled IFN-α (Figure 5).

The procedure was as follows: 200 μl of the specimen was added to each of 2 test tubes in a rack of 20 tubes (Figure 6). Immediately 50 μl of

Figure 4:

"Sucrosep IFN-α IRMA" Boots Celltech Diagnostic Ltd

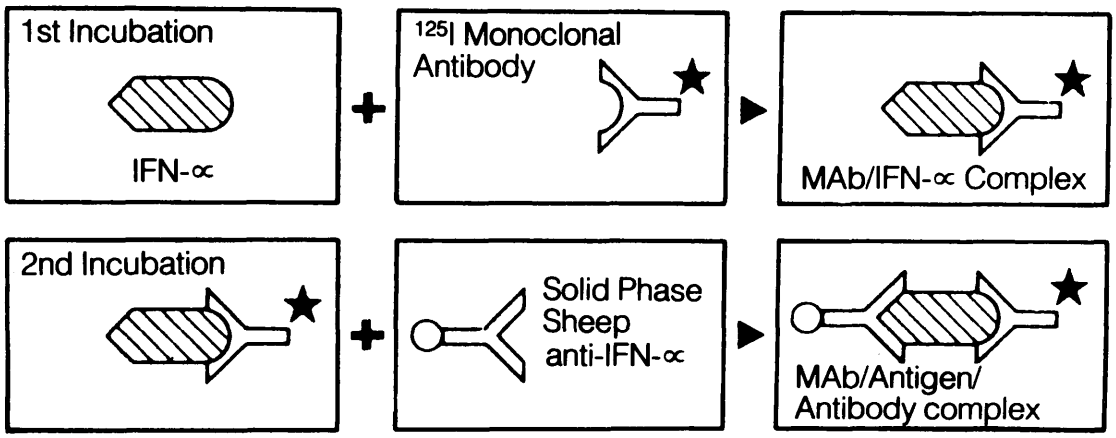


Figure 5:

Principle of the Sucrosep IFN-α IRMA.

Figure 6:

Addition of the specimen to the test tube

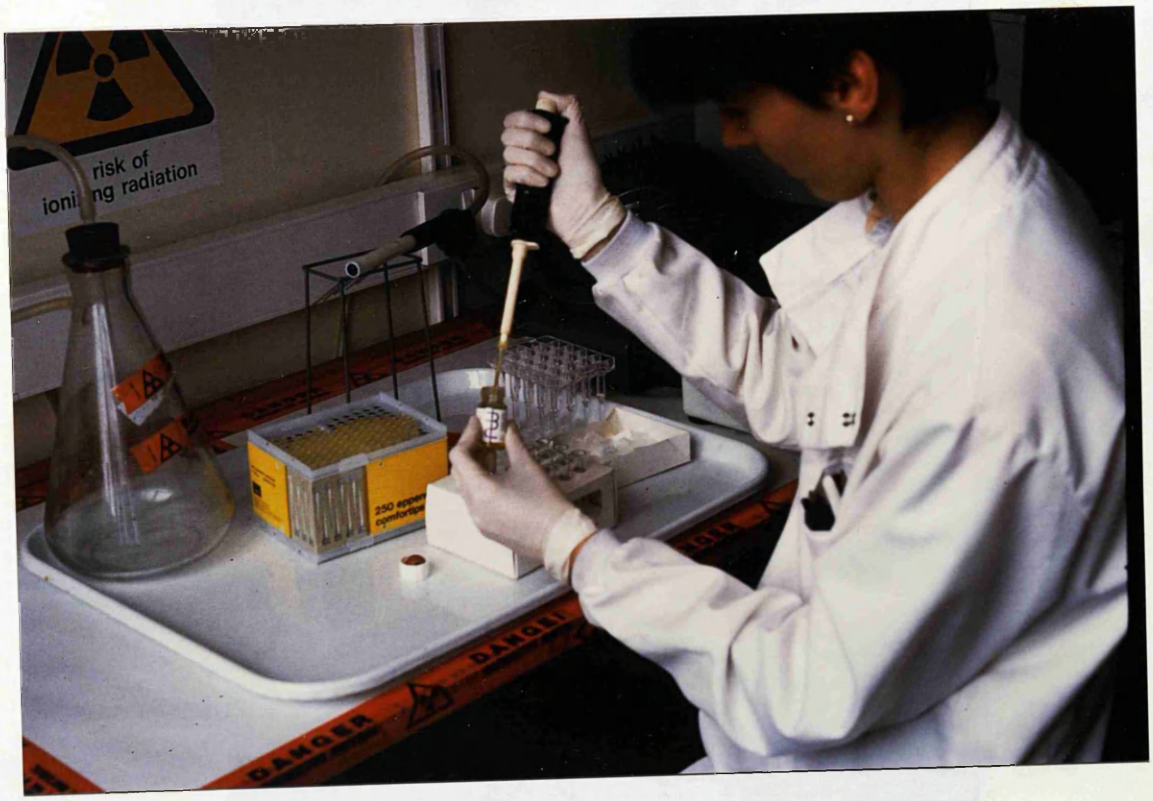


Figure 7:

Addition of the radiolabelled monoclonal antibody to IFN-α

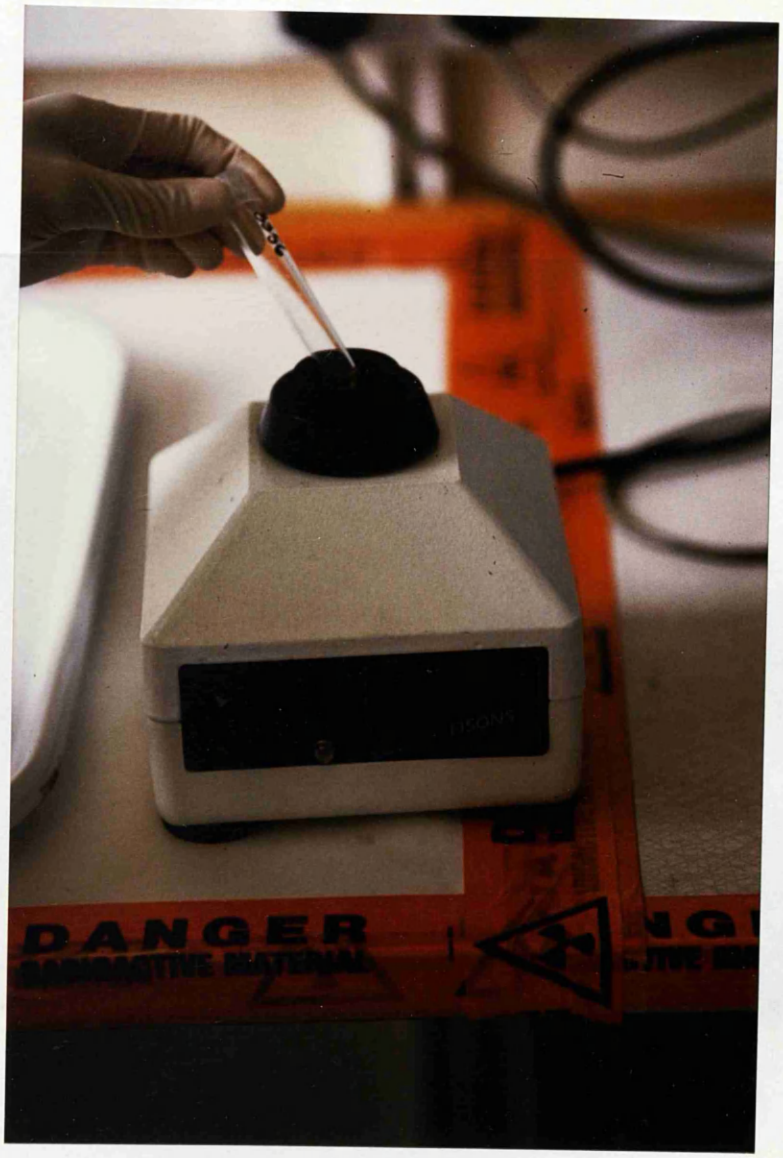


Figure 8:
Vortex-mixture of the specimen and the radiolabelled monoclonal antibody to IFN-α

Figure 9

Addition of the polyclonal antibody to IFN-α

Figure 10:

Repeated inversion of the capped bottle containing the polyclonal antibody IFN-α

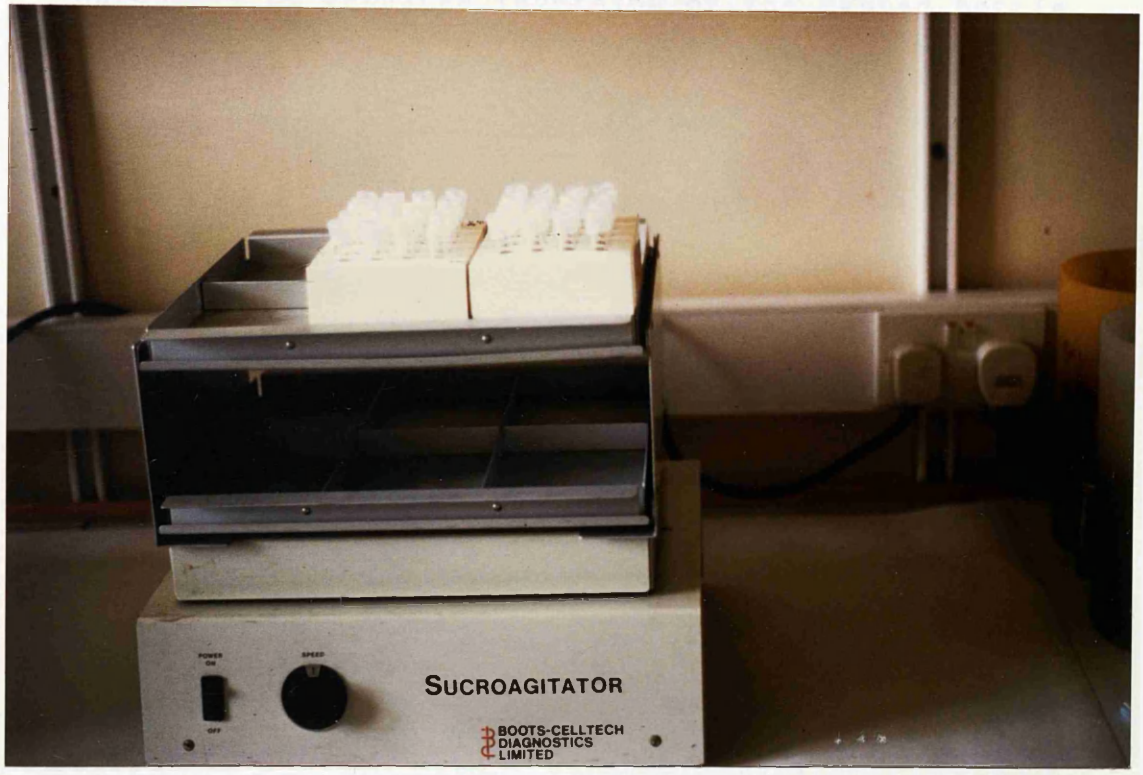


Figure 11:

Orbital shaker to keep the solid phase polyconal antibody in suspension while it reacted with IFN-α present

radiolabelled monoclonal antibody was then added and the tube contents vortex mixed for 60 seconds (Figure 7 and 8).

After an incubation period of 2 hours at room temperature, 50 µl of polyclonal antibody was added to the mixture (Figure 9). The solid phase was mixed thoroughly by repeated inversion of the capped bottle immediately before and during pipetting (Figure 10). Tubes were then placed on an orbital shaker for a further 2 hours in order to keep the solid-phase polyconal antibody in suspension while it reacted with IFN-α present (Figure 11).

At the end of this period the test tubes were removed from the shaker and any empty tube spaces in the racks were filled with spare tubes before commencing the separation procedure. Labelled monoclonal antibody associated with IFN and solid phase was separated and washed by sucrosep non-centrifugation separation system (Wright et al., 1983).

To each tube was added 1 ml of pre-wash buffer to wash the wall of the test tube. The tubes were allowed to stand for five minutes. A sucropette (Figure 12) was placed in each assay tube rack and 2 ml of sucrosep reagent was delivered to lower part of each tube (Figure 13). The dense sucrose solution displaces residual monoclonal antibody and the specimen to the upper part of the test tube. Carefully, the

sucropette was removed when the sucrose solution had been layered below the incubate and it was allowed to stand on the tissue pad in the inverted rack (Figure 14).

The tubes were then left for 15 minutes to allow the solid phase polyclonal antibody to gravitate to the base of the tube, carrying with it any radiolabelled immune complex present.

The contents were aspirated leaving approximately 0.3 ml in the bottom of each tube (Figure 15).

A second sucrose separation step was then carried out after which the tubes were sealed and their radioactivity counted in a gamma scintillation counter (Packard Auto-Gamma 5650, United Technologies, Packard) for a time sufficient to accumulate at least 50,000 counts. A tube with 50 µl of the radio-labelled monoclonal was always added as part of the assay in order to determine the total number of counts.

A summary of the procedure is shown in Figure 16. Using a log/log plot (^{125}I counts versus IFN-α) it was possible to interpolate the IFN-α concentrations of the specimens using a standard curve (Figure 17). It was performed with each new assay kit and in subsequent tests a control of 5 IU/ml was used. The assay standard is a human leucocyte IFN-α preparation which has been calibrated against the MRC

Figure 12:

Sucropette used to deliver the separation solution

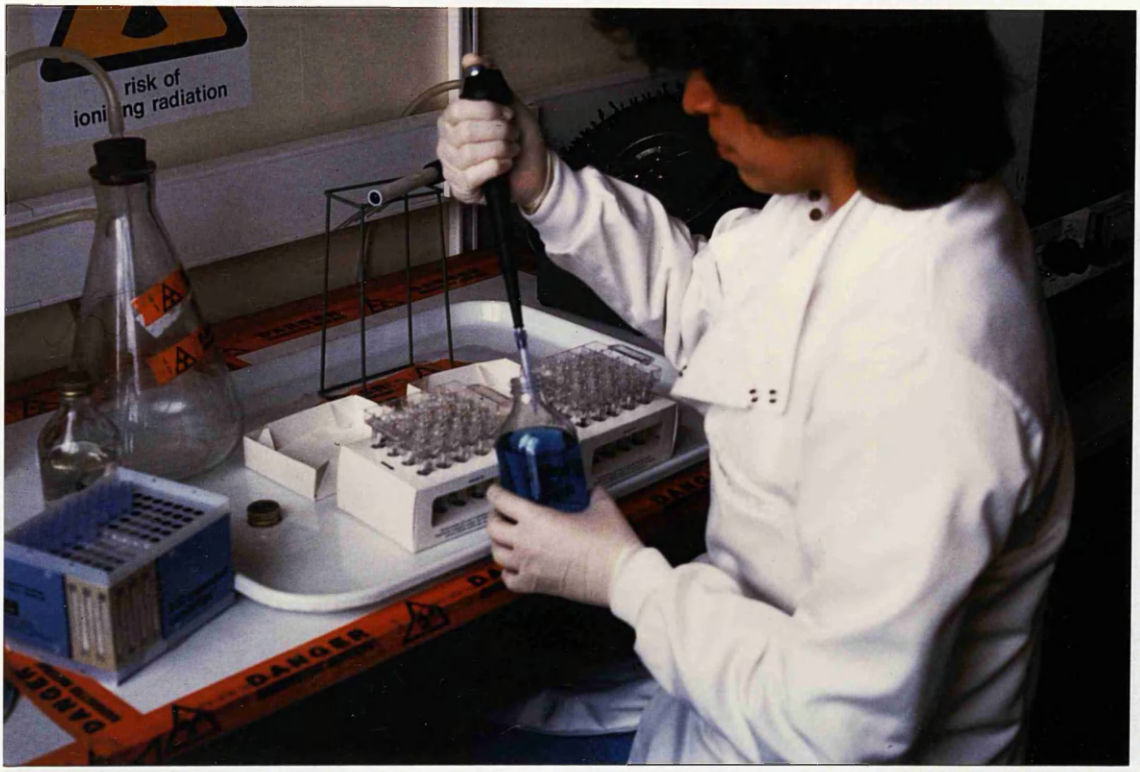


Figure 13:

Addition of the separation solution

Figure 14:

Sucrose solution layering below the incubate

69/19 International Reference Preparation for human leucocyte IFN, obtained from the National Institute of Biological Standards and Control, London, U.K. The IFN-α concentration range covered by the kit is 0-1000 IU/ml.

A standard curve was prepared in VTM using recombinant IFN-α (Appendix 2). Six points in duplicate ranging from 1 to 1024 IU/ml were used. A 1024 IU/ml stock was serially diluted to prepare the standard curve.

Figure 15:

Aspiration procedure

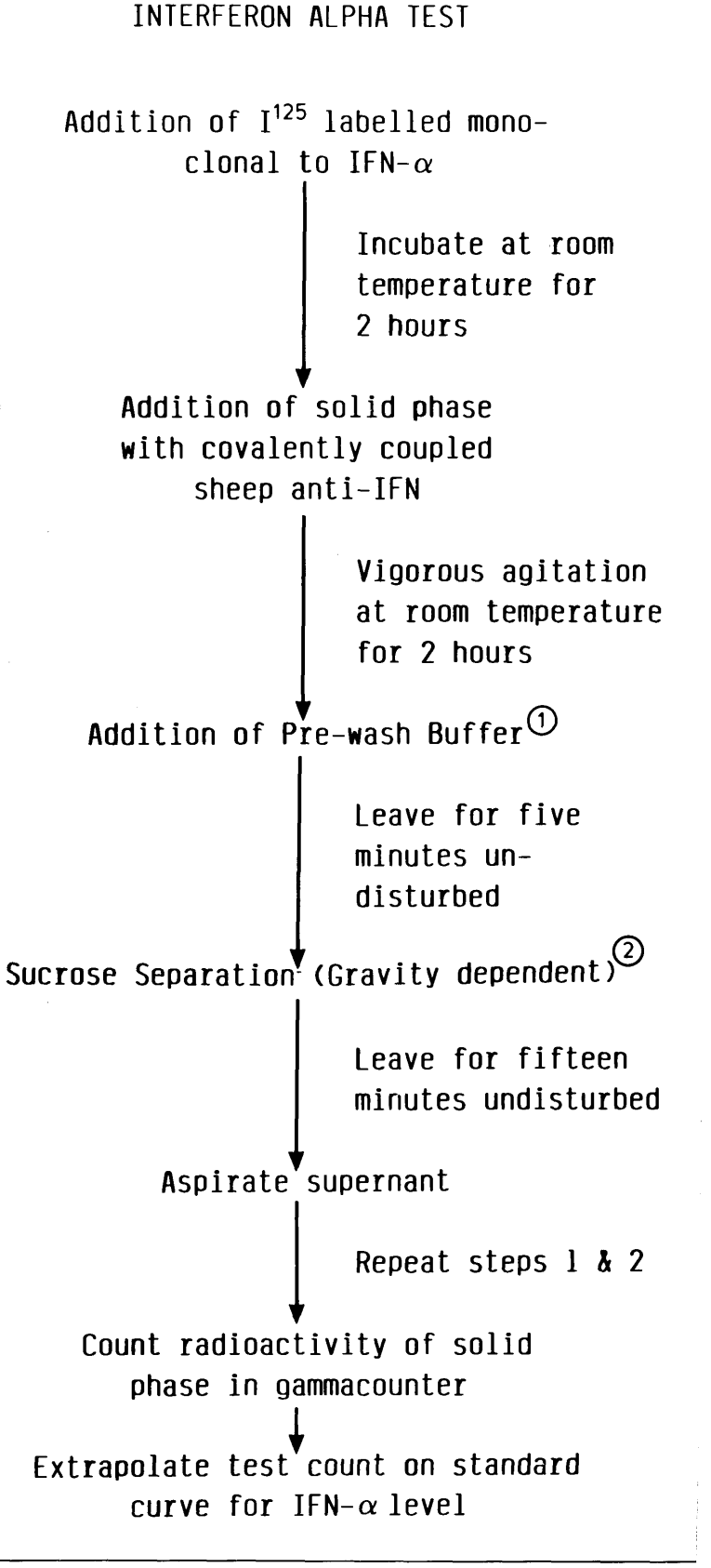


Figure 16:

Summary of the IFN-α procedure

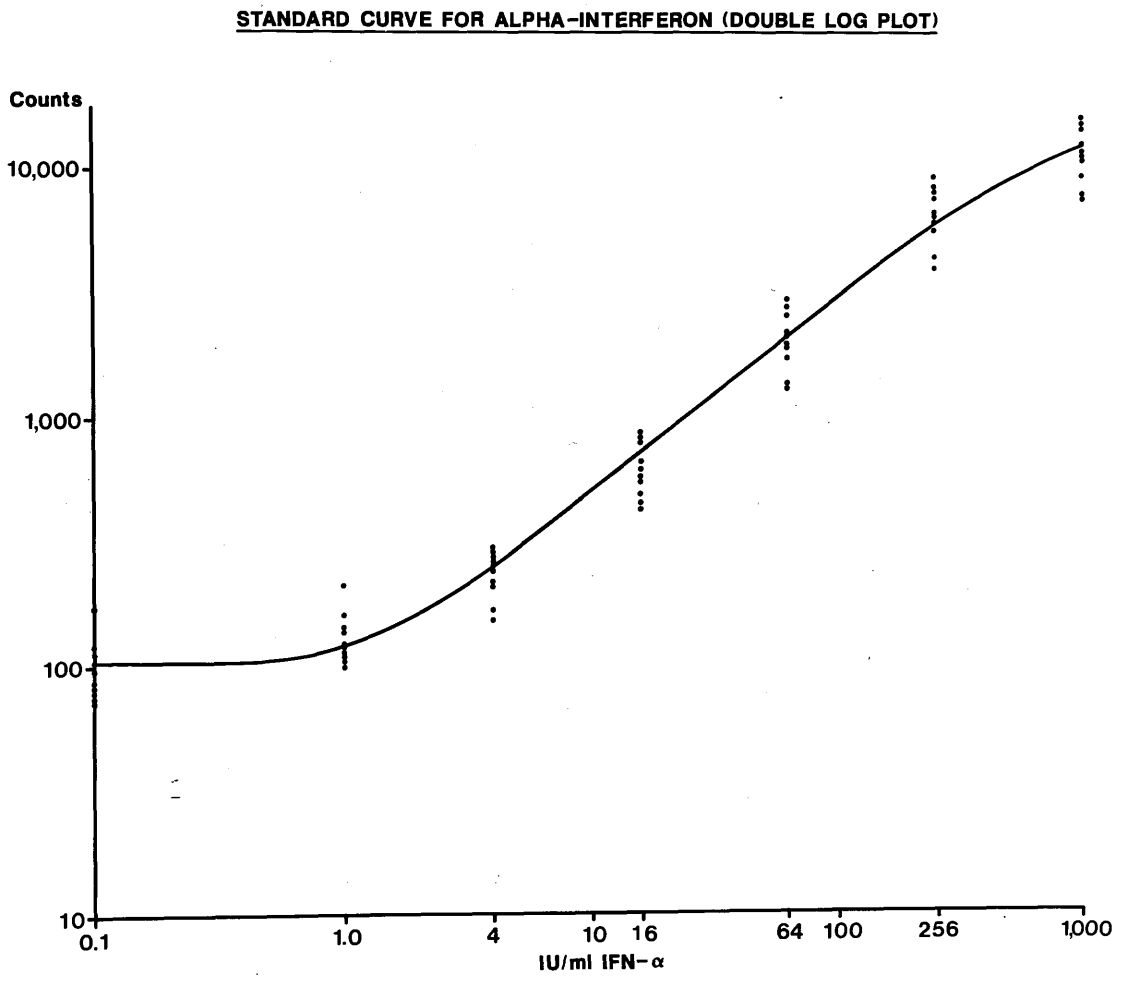

STANDARD CURVE FOR ALPHA-INTERFERON (DOUBLE LOG PLOT)
Counts
10,000
1,000
100
10
0.1
1.0
4
10
16
64
100
256
1,000
IU/ml IFN-α

Figure 17

CHAPTER IV

EXPERIMENTAL SECTION

4.1 PERFORMANCE PARAMETERS OF THE ALPHA INTERFERON IMMUNORADIOMETRIC ASSAY

Sucrosep IFN-α IRMA has been developed by Boots-Celltech Diagnostics for the measurement of human IFN-α in serum or tissue culture fluid. It is the first time that this test has been applied to measure IFN-α in NPS from children with RSV infection and it was considered important to determine the assay performance when VTM was used as a diluent. This was based on the construction of a standard curve which was employed to interpolate IFN-α values. The reproducibility of the test was performed by determining the percentage coefficient of variation when uninoculated fresh VTM were assayed the same day and sensitivity of the test by determining the minimum detection limit to consider a NPS positive for IFN-α by this test. Specificity evaluations were performed by neutralisation-blocking experiments at various concentrations of recombinant and endogenous interferon.

4.1.1 Confidence limits and best-fitting line through a series of points

This experiment demonstrated the

TABLE 2

Statistical values for each point of ten standard curves performed monthly by Sucrosep alpha-interferon immunoradiometric assay

IU/ml	Mean (cpm)	S.D.	S.E.	C.V. (%)
0	101.8	32	10	31
1	132.6	32	10	24
4	243.4	52	16	21
16	642.0	174	55	27
64	2121.4	569	181	27
256	6783.1	1796	568.4	26
1024	12297.5	32	1004.0	26

cpm = counts per minute

S.D. = standard deviation

S.E. = standard error

C.V. = coefficient of variation

TABLE 3

Confidence limits with 95% probability for each point of the standard curve using different concentrations of recombinant alpha-interferon

Standards IU/ml	No. of counts (mean)	No. of counts 95% CL
0	102	79-125
1	133	110-156
4	243	206-281
16	642	518-770
64	2.121	1.712-2.531
256	6.783	5.497-8.069
1024	12.298	10.027-14.569

reproducibility of the test when the mean values for each point of different standard curves performed during a period of ten months were plotted together.

Method

The mean values for each point of different standard curves performed during a period of ten months were employed to calculate the best fit of the curve using a regression equation (Appendix 1).

Confidence limits (95%), standard deviation and standard error for each point were also calculated (Appendix 1).

Results

The best fit of a typical standard curve for the Sucrosep IFN-α IRMA is shown in Figure 17. Although the coefficient of variation is >10% they varied only from 21% to 31% and were very similar for each point (Table 2).

The variability was considered to be acceptable between intra-assay and inter-assay of these curves.

The 95% confidence limits are shown in Table 3.

4.1.2 **Alpha-interferon specificity evaluations of known repetitive controls of virus transport medium with no alpha-interferon activity assayed the same day**

Method

200 μl of VTM was added to each of 2 test tubes. The tubes were labelled from 1 to 20 and

TABLE 4

Alpha-interferon specificity evaluations of known repetitive controls of virus transport medium with no alpha-interferon activity assayed the same day

No. sample	Counts per minute
1	91
2	105
3	88
4	77
5	108
6	104
7	88
8	87
9	102
10	98
11	93
12	86
13	94
14	86
15	86
16	88
17	115
18	95
19	86
20	86

Mean: 93.15
S.D.: 9
C.V.: 9.6%
95% C.L.: 89.2-97.2

samples were assayed for IFN-α as in Section 3.1. The mean of the total number of counts, standard deviation and coefficient of variation were determined (Appendix 1).

Results

Table 4 shows the number of counts per minute of 20 determinations. The mean of total number of counts per minute was 93.2 with a standard deviation of 9 and coefficient of variation of 9.6%. The 95% confidence limits were between 89.2-97.2 (Appendix 1). In a 2 year study a negative control sample did not exceed 102.2 counts per minute.

4.1.3 **Minimum detection limit to consider a nasopharyngeal secretion positive by immunoradiometric assay**

This experiment was carried out to evaluate the sensitivity of the test by determining the cut off level when it is applied to NPS.

Method

IFN-α positive NPS were prepared by a 1/10 dilution of previously tested samples to give the following final concentrations: 0, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 2.0, 4.0 IU/ml. 200 μl of these standard controls was added to each of two tubes/standard and IFN-α was assayed to prepare the endogenous IFN-α standard curve (Set 1). 400 μl of these same standards was added to each of two additional

MINIMUM DETECTION LIMIT OF ENDOGENOUS ALPHA-INTERFERON IN POSITIVE SPECIMENS

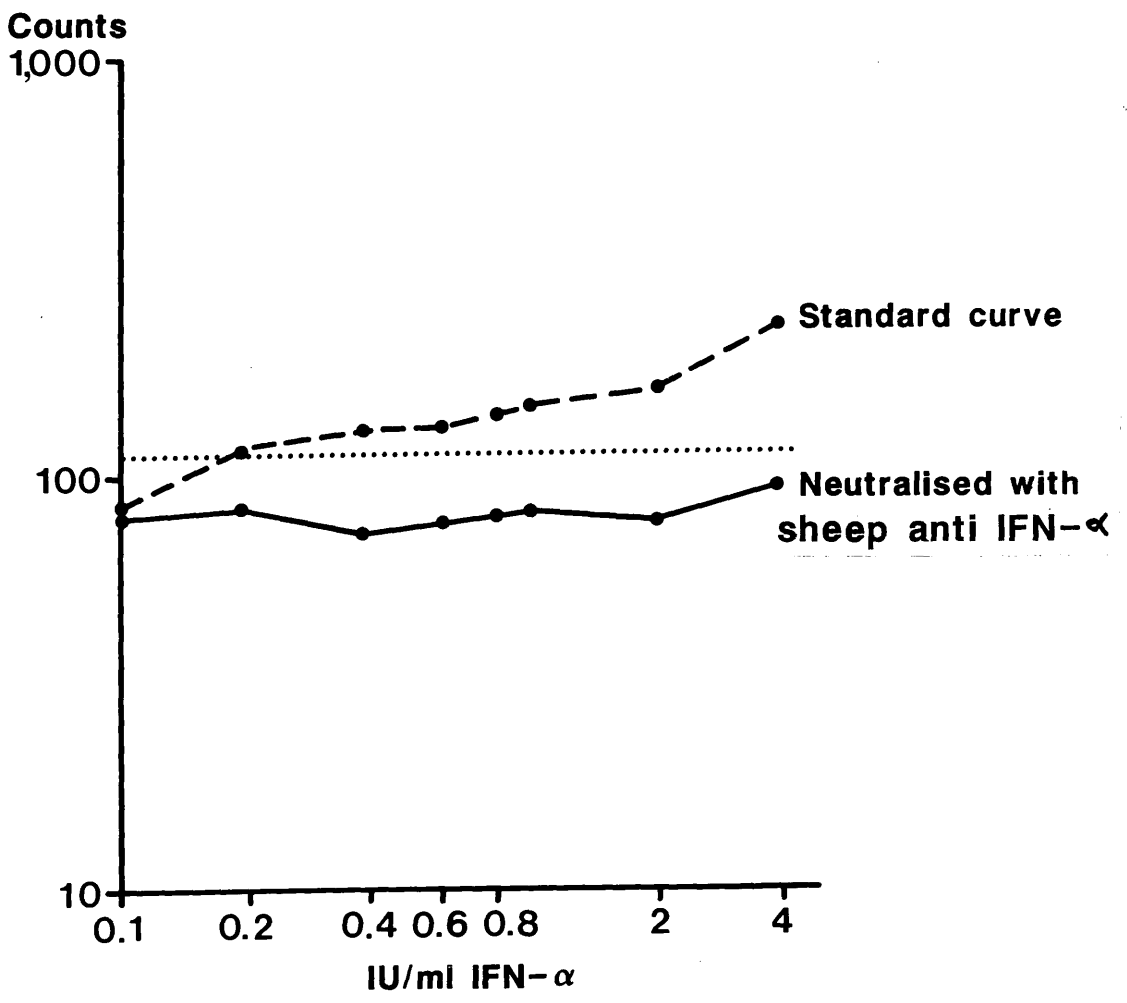


Figure 18

tubes/standard. These tubes containing 400 µl were the samples for neutralization-blocking test (Set 2). Both sets of tubes were labelled with the different levels of IFN-α.

100 µl of sheep anti IFN-α antibody (solid phase) was added to each one of the tubes with the specimens to be neutralized (Set 2). The samples were placed in a orbital shaker and incubated at room temperature for two hours. After this period, the samples were centrifuged and 200 µl of the supernatant was taken and assayed in duplicate for IFN-α activity following the procedure in Section 3.1.

Results

The results of IFN-α detection in control and neutralized-blocked treated samples in a preselected range of NPS is shown in Figure 18. The limits of IFN-α sensitivity in NPS diluted in VTM, capable of being neutralized, is 0.2 IU/ml. After a 1/10 dilution of a NPS, a minimum level of IFN-α in the original sample must be 2 IU/ml by this test.

4.2 EXPERIMENTAL METHODS TO DEMONSTRATE THE PHYSICAL PROPERTIES OF ALPHA-INTERFERON

The use of monoclonal antibodies in immunoassays provide high sensitivity and specificity in detecting IFN in clinical specimens. It was considered important to evaluate some of the properties which characterized IFN-α and to assess the ability of

the Sucrosep IFN-α IRMA in measuring differences during IFN inactivation.

Using recombinant IFN-α and NPS from patients with proven RSV infections and prepared as described in Section 2.1.2, selected samples were used to evaluate some of the properties of endogenous IFN-α i.e. heat and trypsin inactivation.

Methods and Results

4.2.1 **HEAT INACTIVATION EXPERIMENT**

The experiment attempts to determine whether exposure at 56^{o}C of several standards of recombinant IFN-α and two different levels of IFN-α in NPS will produce a change in activity when it is compared with a control curve.

Heat-inactivation curve of recombinant alpha interferon

Method

IFN standards of 20, 100, 1000 IU/ml of recombinant IFN-α were prepared from the standard preparation of 1024 IU/ml. In order to prepare two sets of tubes in duplicate, 200 μl of each IFN standard (20, 100, 1000 IU/ml) was added to each of 4 test tubes. The tubes for inactivation were labelled with the different times to be tested (10, 20, 30, 40, 50, 60 minutes).

The rack of tubes was placed in a water bath and the standards were heated to 56^{o}C at the time listed above. The IFN-α assay was performed for the

control and heat treated standards using the same procedure as Section 3.1.

Results

The difference in international units per millilitre between the control and the heat treated recombinant IFN-α of 20 IU/ml was calculated and plotted against time in minutes (Figure 19).

There was partial inactivation with a loss of 15 IU/ml IFN-α (75% loss of IFN-α activity) at the end of the experimental time. Inactivation became evident after 20 minutes of treatment and continued to 60 minutes when the test was terminated.

Heat-inactivation at the same temperature and using recombinant IFN-α of 100 IU/ml is shown in Figure 20. There was also partial inactivation with a loss of 50 IU/ml (50% loss of IFN-α activity) after 60 minutes treatment. Again an abrupt fall at 20 minutes of heat treatment was seen.

Heat-inactivation of a standard of 1000 IU/ml of recombinant IFN-α versus time in minutes is shown in Figure 21. A signficant inactivation was seen after 20 minutes which continued throughout the period of heat treatment to 60 minutes. At 60 minutes, 44% of IFN-α activity remained (56% loss of IFN-α activity).

In these three experiments with recombinant IFN-α partial inactivation of IFN-α activity was seen

HEAT INACTIVATION CURVE AT 56°C USING A STANDARD OF 20 IU/ml OF RECOMBINANT ALPHA-INTERFERON

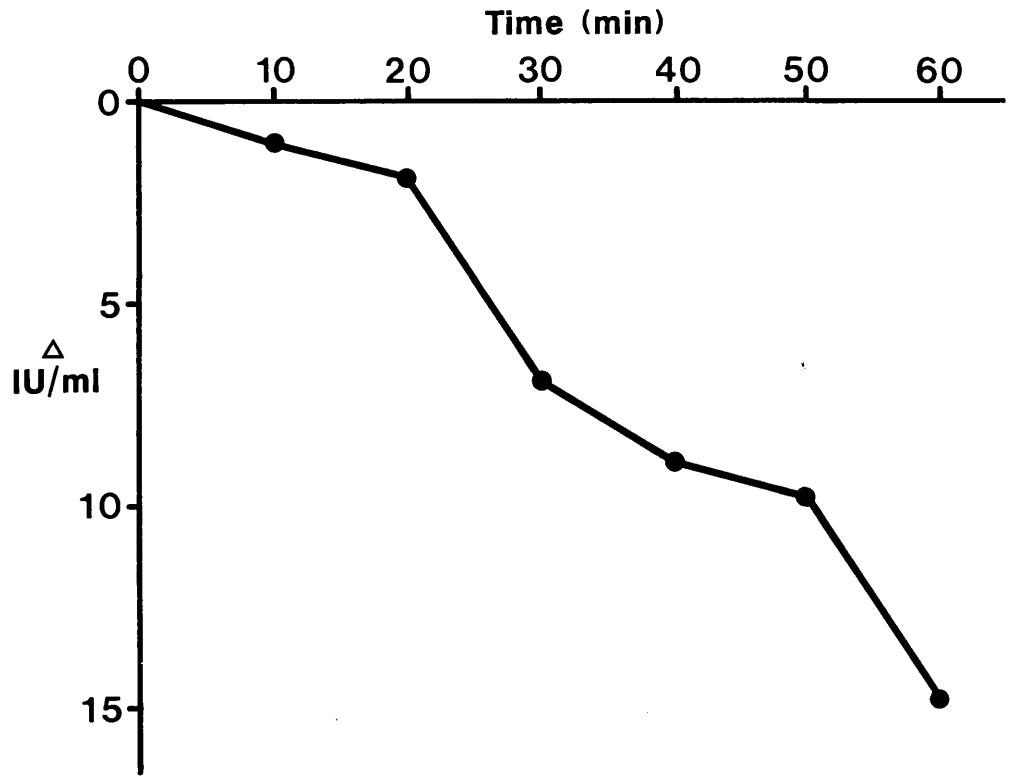


Figure 19

HEAT INACTIVATION CURVE AT 56°C USING A STANDARD OF 100 IU/ml OF RECOMBINANT ALPHA-INTERFERON

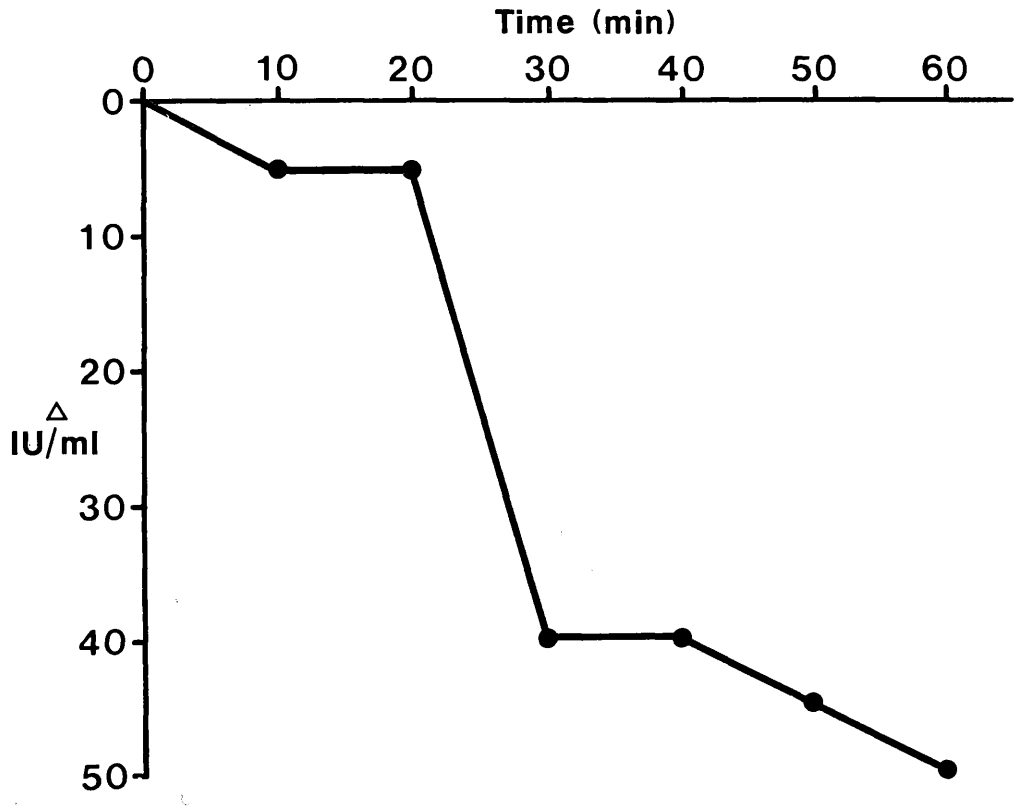


Figure 20

HEAT INACTIVATION CURVE AT 56°C USING A STANDARD OF 1000 IU/ml OF RECOMBINANT ALPHA-INTERFERON

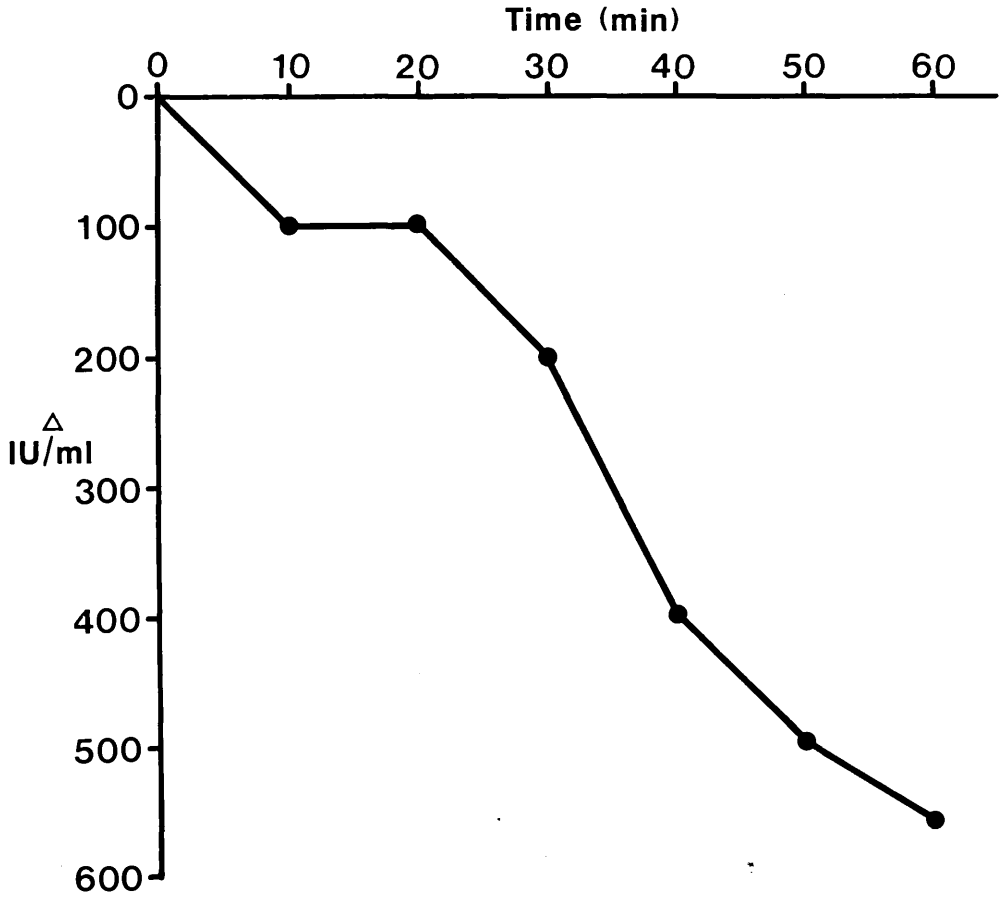


Figure 21

TABLE 5

Loss of recombinant alpha-interferon activity
after heat treatment at 56°C

Percentage loss of IFN-α activity				Total
IFN-α IU/ml	0-20 min	21-40 min	41-60 min	0-60 min
20	10%	35%	30%	75%
100	5%	35%	10%	50%
1000	10%	30%	16%	56%

by treatment with heat at 56°C for 60 minutes. A two stage inactivation was seen with a little loss of activity up to 20 minutes treatment (5-10% loss) followed by a steady loss of activity (50-75%) to 60 minutes treatment when the experiments were terminated. This two stage inactivation appeared constant over the IFN-α concentration range of 20-1000 IU/ml (see Table 5). The percentage of loss of IFN-α activity in the three periods were calculated as follows:

$$\% \text{ loss of IFN-}\alpha \text{ activity (0-20)} = \frac{\Delta \text{ IU/ml (20 min)} \times 100}{\text{rIFN-}\alpha \text{ IU/ml}}$$

$$\% \text{ loss of IFN-}\alpha \text{ activity (21-40)} = \frac{\Delta \text{ IU/ml (40)} - \Delta \text{ IU/ml (21)} \times 100}{\text{rIFN-}\alpha \text{ IU/ml}}$$

$$\% \text{ loss of IFN-}\alpha \text{ activity (41-60)} = \frac{\Delta \text{ IU/ml (60)} - \Delta \text{ IU/ml (41)} \times 100}{\text{rIFN-}\alpha \text{ IU/ml}}$$

Heat-inactivation of two different levels of alpha interferon in nasopharyngeal secretions

Method

Two pools of NPS with levels of IFN at 52 and 100 IU/ml were prepared. The IFN-α assay was performed to determine the exact value of the pool preparation. In order to prepare two sets of tubes, 200 μl of each pool (52 and 100 IU/ml) was added to each of 4 test tubes.

HEAT INACTIVATION OF A POOL OF POSITIVE SPECIMENS AT A CONCENTRATION OF 52 IU/ml

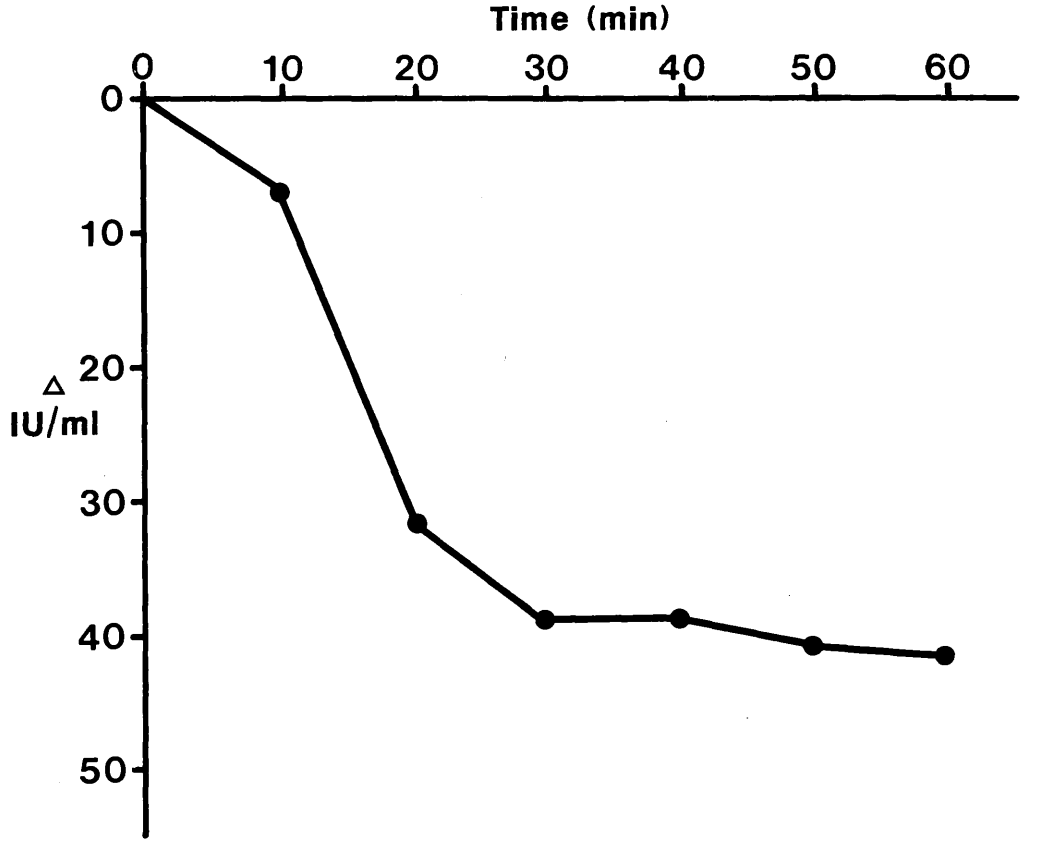


Figure 22

HEAT INACTIVATION OF A POOL OF POSITIVE SPECIMENS AT A CONCENTRATION OF 100 IU/ml

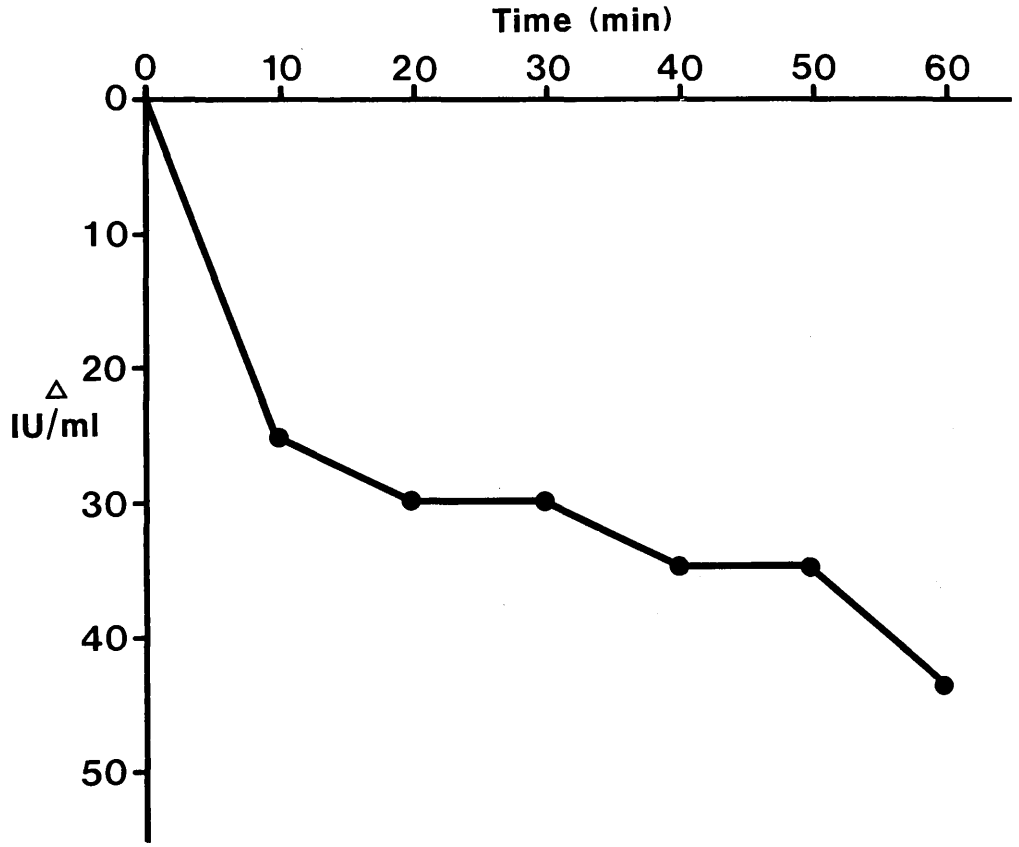


Figure 23

The tubes for inactivation were labelled with the different temperatures to be tested (10, 20, 30, 40, 50, 60 minutes). The rack of tubes was placed in a water bath and the samples were heated to 56°C at the time listed above.

IFN-α was assayed for the control and inactivated specimens using the same procedure as Section 3.1.

Results

The difference in IFN-α activity of a pool of positive NPS at a concentration of 52 IU/ml was tested during a period of sixty minutes at 56°C (Figure 22).

There was partial inactivation of IFN-α activity with loss of 32 IU/ml (64% loss of IFN-α activity) in the first period of 0-20 minutes and a further loss of 9 IU/ml (19% loss of IFN-α activity) in the second period (20-60 minutes).

A pool of positive NPS at a concentration of 100 IU/ml was also made and tested. The loss of IFN-α activity in international units per millilitre during heat inactivation is shown in Figure 23. Similar to results obtained with the previous pool the fall in activity from 0-20 minutes was a loss of activity of 30 IU/ml (30% loss of IFN-α activity) followed by a slower decrease in inactivation, i.e. a loss of 14 IU/ml (14% loss of IFN-α activity between 20-60

TABLE 6

Loss of endogenous alpha-interferon activity after heat treatment at 56°C

Percentage loss of IFN- activity				Total
IFN- IU/ml	0-20 min	21-40 min	41-60 min	0-60 min
52	64%	12%	7%	83%
100	30%	5%	9%	44%

minutes of heat inactivation).

A two stage partial inactivation of IFN-α activity was seen when endogenous IFN-α was heat treated. In the first period of 20 minutes a 30-64% loss of IFN-α activity was seen, followed by a further 5-12% loss in the second period of 40 minutes. Although, a two stage partial inactivation by heat treatment was seen for both recombinant and endogenous IFN, the rate of inactivation of endogenous IFN was higher in the first 20 minutes compared to recombinant IFN-α (see Tables 5 and 6). Although inactivation of recombinant IFN-α appeared to be resisted in the first 20 minutes, a marked fall in IFN-α activity in the second 20 minute period i.e. between 20-40 minutes of heat inactivation was seen (see Tables 5 and 6). The loss of activity in the three periods were calculated as follows:

$$\%\ \text{loss of IFN-}\alpha\ \text{activity (0-20)} = \frac{\Delta\ \text{IU/ml (20)} \times 100}{\text{eIFN-}\alpha\ \text{IU/ml}}$$

$$\%\ \text{loss of IFN-}\alpha\ \text{activity (21-40)} = \frac{\Delta\ \text{IU/ml (40)} - \Delta\ \text{IU/ml (21)} \times 100}{\text{eIFN-}\alpha\ \text{IU/ml}}$$

$$\%\ \text{loss of IFN-}\alpha\ \text{activity (41-60)} = \frac{\Delta\ \text{IU/ml (60)} - \Delta\ \text{IU/ml (41)} \times 100}{\text{eIFN-}\alpha\ \text{IU/ml}}$$

TRYPSIN INACTIVATION CURVE USING THREE DIFFERENT STANDARDS OF RECOMBINANT ALPHA-INTERFERON

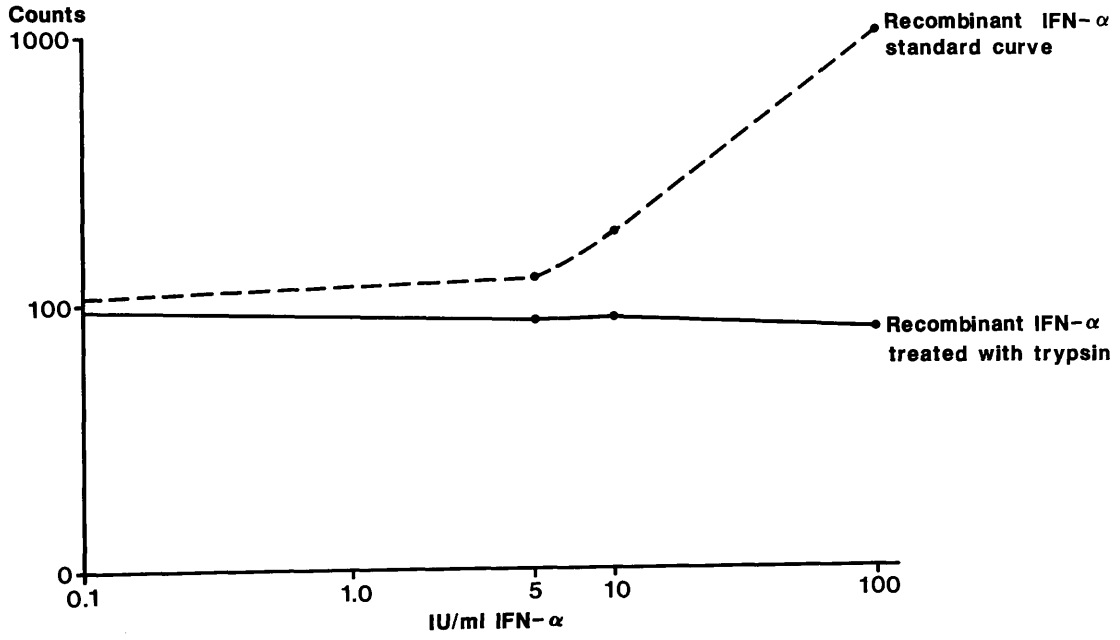


Figure 24

4.2.2 TRYPSIN INACTIVATION

The purpose of this experiment is to determine the effect of trypsin over the protein-content of IFN-α present in NPS and recombinant IFN-α standards compared to a control curve.

Trypsin inactivation curve of recombinant alpha interferon

Method

IFN standards of 5, 10 and 100 IU/ml of recombinant IFN-α were prepared from a standard preparation of 1024 IU/ml. In order to prepare two sets of tubes, 200 µl of each standard (5, 10, 100 IU/ml) and 200 µl of trypsin (see Appendix 2) was added to each of 2 test tubes/standard.

The rack of tubes was placed in a water bath for one hour at 37°C. After this period 100 ul of fetal calf serum was added in order to neutralize the trypsin. IFN-α assay was performed using the same procedure as Section 3.1.

Results

Recombinant IFN-α was found to be trypsin sensitive when the same standard curve without trypsin was employed. It was possible to inactivate 100% of the activity by trypsin in all the standards of recombinant IFN-α (Figure 24).

Trypsin inactivation of three different levels of alpha interferon in nasopharyngeal secretions

Method

Three pools of specimens with IFN-α levels of 1.0, 1.5 and 7.5 IU/ml were prepared. The IFN-α assay was performed (as Section 3.1) to determine the exact value of the pool preparation. In order to prepare two sets of tubes, 200 µl of each pool and 200 µl of trypsin were added to each of two test tubes/standard. The rack of tubes was placed in a water bath for one hour at 37°C. After this period 100 µl of fetal calf serum was added in order to neutralize the trypsin.

IFN-α assay was performed using the same procedure as Section 3.1.

Results

Again, trypsin inactivation was obtained using endogenous IFN-α (Figure 25).

It was possible to demonstrate inactivation of the endogenous IFN-α activity in the three pools of specimen/standards.

As expected, for the properties of IFN-α, trypsin treatment completely inactivated both recombinant and endogenous IFN-α as measured by this sensitive assay.

TRYPSIN INACTIVATION OF ALPHA-INTERFERON ACTIVITY OF THREE POOLS OF POSITIVE SPECIMENS

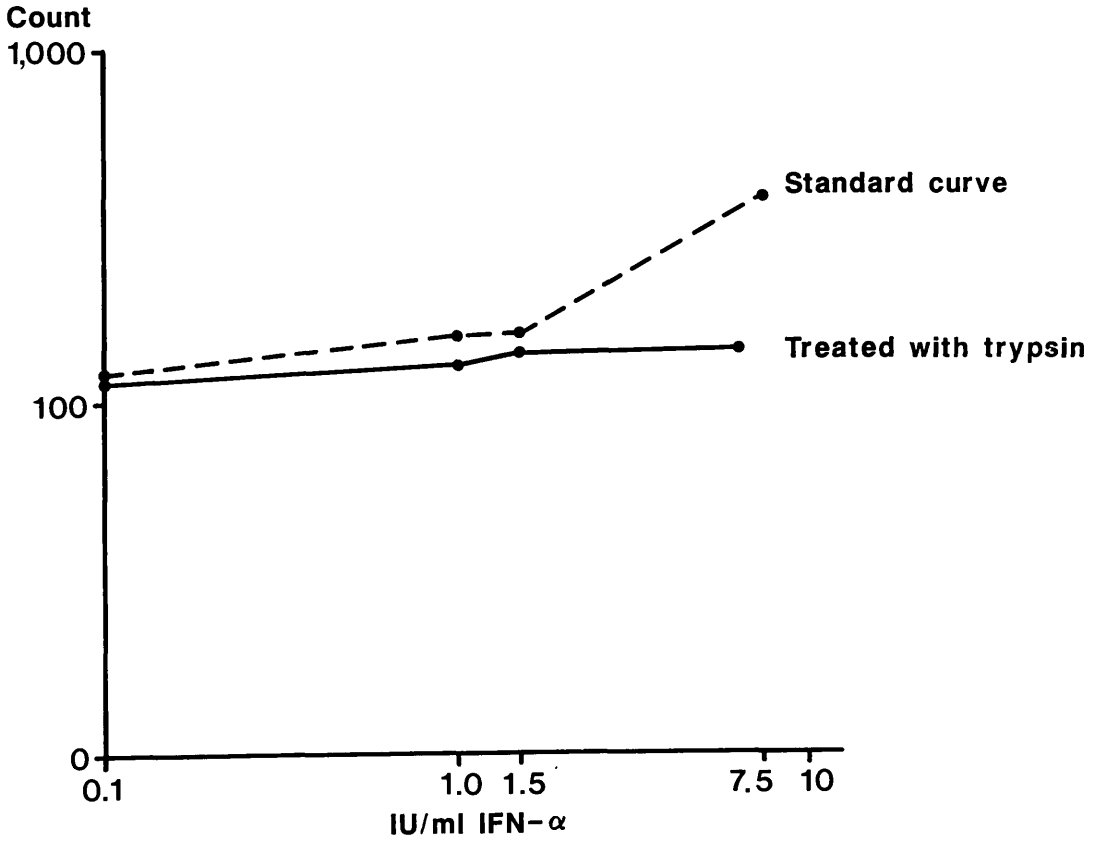


Figure 25

4.3 EXPERIMENTAL METHODS TO DEMONSTRATE THE SPECIFICITY OF ALPHA-INTERFERON

4.3.1 NEUTRALIZATION-BLOCKING EXPERIMENT

This experiment evaluates the specificity of the test when sheep anti IFN-α is used to block any IFN-α present in the standards and specimens. After a blocking step, samples were tested for any remaining IFN-α activity.

Neutralization-blocking of recombinant alpha-interferon activity

Method

IFN standards of 20, 100, 1000 IU/ml of recombinant IFN were prepared from the standard preparation of 1024 IU/ml. 400 µl of each standard was added to each of 2 tubes/standard. These were the samples for neutralization-blocking experiments. Both sets of tubes were labelled with the different levels of IFN-α,

100 µl of sheep anti IFN-α antibody (solid phase) was added to each one of the tubes with the specimens to be neutralized.

The samples were placed in an orbital shaker and incubated at room temperature for two hours.

After this period, the samples were centrifuged and 200 µl of the supernatant was taken and assayed in duplicate for IFN-α activity following the procedure in Section 3.1. At the same time IFN-α was

NEUTRALISATION BLOCKING OF ALPHA-INTERFERON ACTIVITY OF THREE DIFFERENT RECOMBINANT ALPHA-INTERFERON STANDARDS

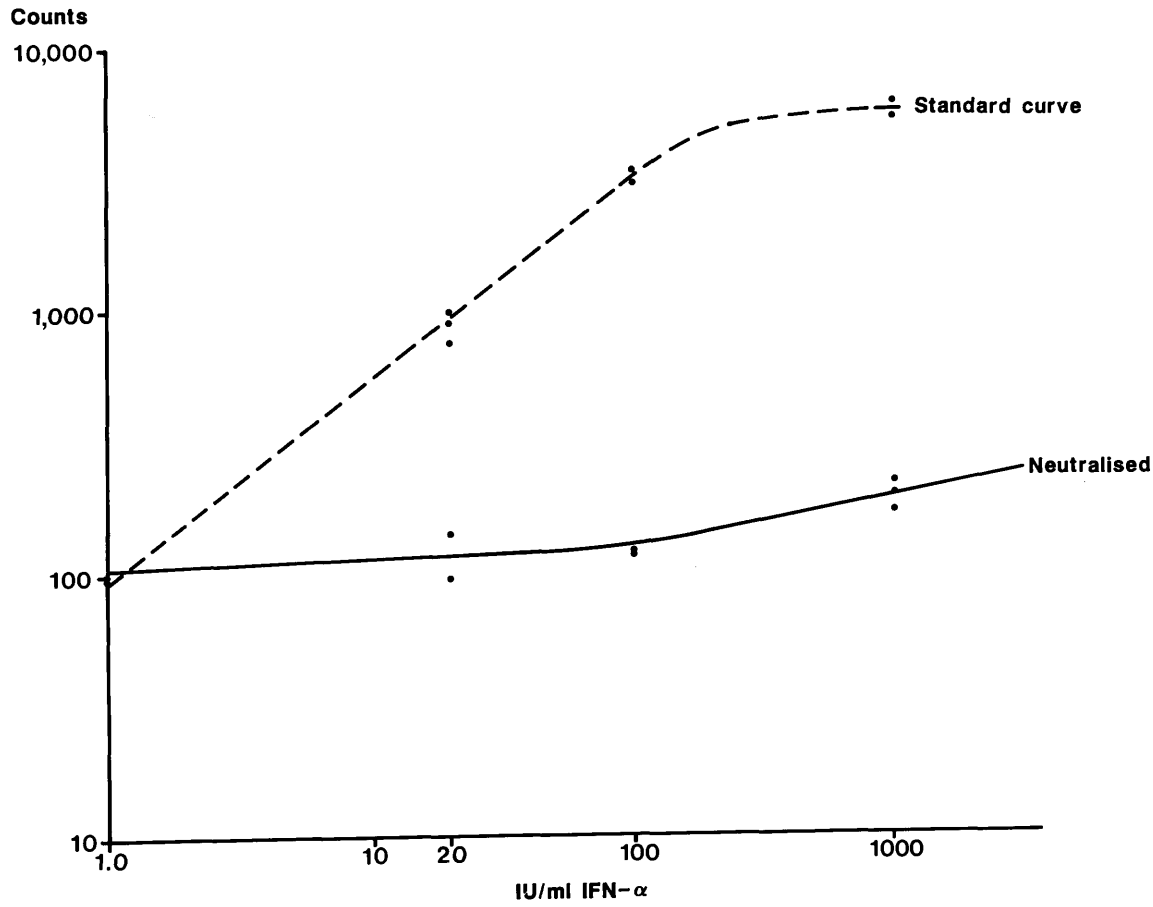


Figure 26

assayed for the control samples.

The solid phase in this experiment was a sepharose bead system where sheep anti IFN-α was covantly bound. It was possible to capture IFN-α onto the solid phase with incubation and remove it from the specimen by centrifugation. The supernatant could then be tested for residual unblocked IFN-α .

Results

It was possible to neutralize -block 100% of the IFN-α activity in the 20 and 100 IU/ml standards of recombinant IFN-α (Figure 26), but residual IFN-α activity (2.8 IU/ml (0.28%)) remained in the 1000 IU/ml control after blocking.

Neutralization of endogenous alpha-interferon activity (range 1-15 IU/ml) in nasopharyngeal secretions by sheep anti alpha-interferon

Method

Five pools of RSV positive NPS with levels of IFN-α at 1.0, 1.2, 4, 4.6, and 15 IU/ml were prepared by appropriate dilution and repeat testing.

400 μl of each pool was added to each of 2 test tubes. 100 μl of sheep anti IFN-α antibody (solid phase) was added to each of the tubes to be neutralize-blocked. The samples were placed in an orbital shaker and incubated at room temperature. The samples were centrifuged and 200 μl of each supernatant was taken and assayed for IFN-α activity following the

NEUTRALISATION - BLOCKING OF ENDOGENOUS ALPHA-INTERFERON ACTIVITY (RANGE 1-15 IU/ml) IN NASOPHARYNGEAL SECRETIONS BY SHEEP ANTI ALPHA-INTERFERON

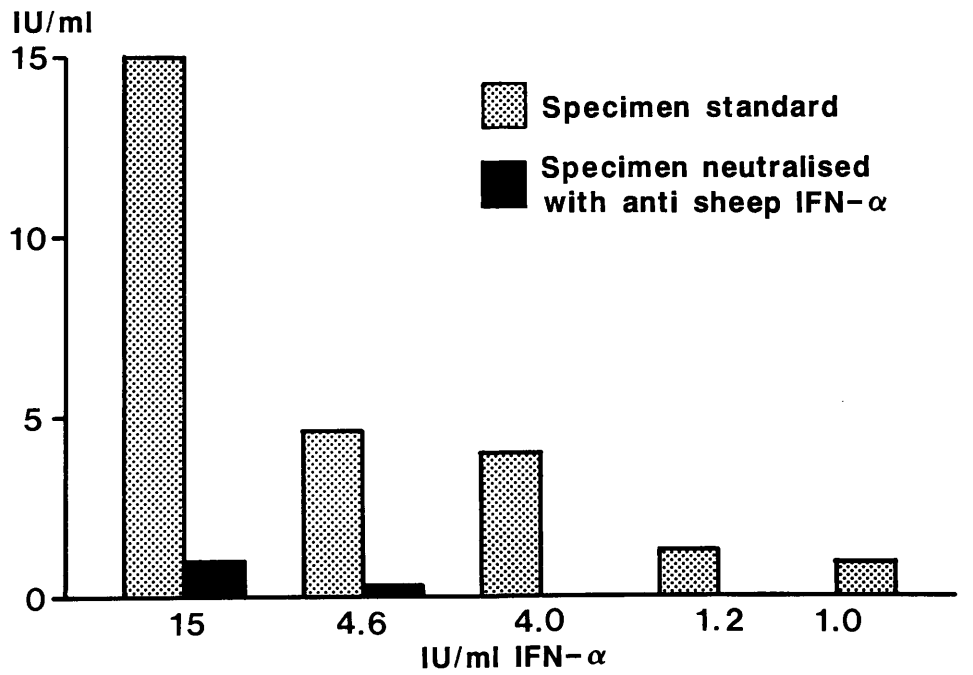


Figure 27

procedure in Section 3.1. IFN-α was assayed for the control samples.

Results

Neutralization-blocking of IFN-α activity of five pools of NPS specimens with levels of 1.0, 1.2, 4, 4.6 and 15 IU/ml is shown in Figure 27.

It was possible to neutralize -block 100% of the activity in three of the five pools of specimens (1.0, 1.2, and 4 IU/ml).

In the pool of specimens of 4.6 IU/ml and 15 IU/ml the percentage of neutralization-blocking was 93.5% and 93% of IFN-α activity respectively.

This experiment confirmed the specificity of the Sucrosep IFN-α IRMA by significantly reducing IFN-α levels by treatment with anti-IFN-α antibody.

CHAPTER V

5.1 DISCUSSION OF THE EXPERIMENTAL SECTION

In a prospective evaluation of any new method to determine IFN-α levels in a clinical study, it is important to determine the performance of the test in terms of sensitivity and specificity parameters with special reference to the clinical specimens to be examined. In previous studies specificity testing has taken account of the test's ability to demonstrate the properties of interferon in the clinical specimens. In this study using 'Sucrosep IFN-α IRMA' clinical specimens were subjected to heat stability and lability to trypsin for this purpose. Attempts were also made to determine pH lability but insufficient material was available for this study.

The reproducibility of the test was evaluated when the mean values for each point of different standard curves were plotted together (Figure 17). A standard curve was used to interpolate IFN-α values for unknown samples of NPS. The variability found between the standard curves of different kits of reagents were very similar for each point and varied from 21% to 31% (Table 2). This is a contrast to the

biological assays wherein interassay coefficient of variations are often greater than 50% (Protzman et al., 1985).

The interassay precision of the test was also evaluated when uninoculated fresh samples of VTM were assayed the same day giving a coefficient of variation of 9.6% (Table 4). This is in agreement with previous reports using the same assay. Ho-Yen et al., 1987, found that the coefficient of variation was <10% and Protzman et al., 1985, <6%.

The sensitivity of Sucrosep IFN-α IRMA for NPS was also evaluated and the test was able to detect as little as 0.2 IU/ml in a diluted clinical specimen (Figure 18).

Although the use of monoclonal antibodies in immunoassays provides high sensitivity and specificity in detecting IFN, it was considered important to assess the ability of the Sucrosep IFN-α IRMA to measure differences before and after IFN inactivation. In the present study, partial inactivation by treatment with heat at 56^{o}C was found when either recombinant IFN-α or a pool of clinical specimens containing endogenous IFN-α were analysed. Inactivation became evident after 20 minutes of treatment and continued to 60 minutes when the test was terminated. In the three experiments performed with standard preparations, a two stage inactivation was seen with little loss of

activity in the first 20 minute period of treatment (5-10% loss) followed by a steady loss of activity (50-75%) to 60 minutes treatment (Table 5). When the same experiment was applied to two different levels of IFN-α in NPS, again a two stage of inactivation of endogenous IFN-α was seen, but in this case the rate of inactivation of endogenous IFN-α was higher in the first 20 minutes and a slower rate between 20-60 minutes treatment compared to the inactivation rate of recombinant IFN-α (Tables 5 and 6).

It is likely that IFN present in NPS is a mixture of IFN-α subtypes whereas the standard preparation is a recombinant monotypic IFN-α. Differences in the subtype composition, chemical structure and buffering characteristics in suspending fluids may give rise to differences in the inactivation curves seen.

In order to confirm the protein characteristics of IFN-α, standard preparations of recombinant IFN-α and clinical specimens containing endogenous IFN-α were heated with trypsin for trypsin sensitivity. In these experiments, trypsin treatment completely inactivated both recombinant and endogenous IFN-α (Figures 24 and 25). In similar experiments using biological assays McIntosh, 1978 found that trypsin treatment produced a 4-64 fold reduction in capacity of IFN in five secretions positive for

influenza virus. Heating at 56°C for 30 minutes destroyed activity in only one secretion. On the other hand, Wheelock et al., 1964 found complete inactivation by trypsin treatment and partial inactivation by heating 9 sera samples at 56°C (McIntosh, 1978; Wheelock et al., 1964).

In this study neutralization-blocking experiments were performed in order to demonstrate the specificity of the Yok 5/19 monoclonal antibody in detecting IFN-α . In both cases using standards and specimens, it was possible to neutralize almost all of the IFN-α activity confirming excellent specificity in this test (Figures 26 and 27). Previous studies of IFN in NPS have used biological assays which are not always practical for routine application. Biological assays are labour intensive, slow and not many samples can be tested at once. Depending on the cell line and virus used in the assay, these assays detect total IFN i.e. alpha plus beta plus gamma. Further tests are required to differentiate a particular IFN type (Protzman et al., 1985). The radioimmunometric assay used for this study was rapid, reproducible, sensitive and specific to IFN-α and the results were obtained within 24 hours. Prior to use of the 'Sucrosep IFN-α IRMA' to detect IFN-α in clinical specimens, any particular matter present in the specimens such as mucus or debris was removed by centrifugation. Virus

transport medium was used as a diluent and in order to maintain reproducibility, a VTM blank control was used every time that the test was performed.

Samples were stored at -70°C after collection and when an appropriate number was accumulated the test was carried out. Repeated freezing and thawing of the specimens was also avoided by multiple aliquoting of homogenised samples. The stability of IFN varies considerably according to the type of IFN, the species of origin and the degree of purification, but IFN preparations can be stored for many months at -20°C or -70°C without loss of activity (McNeill, 1981).

The amount of NPS collected was usually very small 0.1-0.5 ml and it was considered necessary to dilute the NPS 1:10 in VTM prior to testing. This gave sufficient material for the other viral studies undertaken.

The assay kit employed in this study to determine IFN-α in NPS contains radioactive material. All the tests were carried out within a designated area with monthly monitoring of the level of radioactivity present on laboratory surfaces and equipment. A record of the amount of radioactivity in microcuries (uCi) was kept and sent to the regulatory authorities at the RHSC. One of the disadvantages of ^{125}I is that it has half life of 59.6 days which gives a limitation of time in which the test can be used.

A number of features in the 'Sucrosep IFN-α IRMA' have been brought together to bring sensitivity and specificity with ease of handling. It is clearly advantageous to use a non-competitive monoclonal antibody together with a conventional hyperimmune antisera in a sandwich technique. In this way a monoclonal antibody can be selected for use as radiolabelled tracer (ensuring binding purity) allowing the hyperimmune sera with multiple epitopes attached to a solid phase to bring down the immune complexes formed with the monoclonal antibody (Hunter et al., 1983). This solid phase separated easily and settled fairly quickly under unit gravity to allow its use in the sucrose solution. This gave the test both speed and efficiency (Wright et al., 1982).

It is clear from these studies that this assay is well devised and was reproducible, rapid, sensitive and specific in the quantitative and qualitative evaluation of IFN-α in clinical samples. The test was found to be highly satisfactory for use with NPS in this study.

CHAPTER VI

6.1. RESULTS OF THE CLINICAL ASPECTS

6.1.1. EPIDEMIOLOGY OF RESPIRATORY INFECTION IN GLASGOW

During October 1985 and March 1986, 208 children were admitted with suspected respiratory infection to the Royal Hospital for Sick Children (RHSC), Glasgow which is the main paediatric centre in Scotland. The number of respiratory admissions in children less than 15 years at the RHSC, Southern General (SGH) and Ruchill Hospitals from June 1985 to May 1986 is shown in Table 7. From the total number of respiratory admissions the RHSC admitted 1903 children (79.5%). Paediatric departments at Ruchill and SG Hospitals have annual admissions for respiratory infections of 188 (7.8%) and 302 (12.6%) children respectively.

When the same analysis was made for children less than four years for the same period of time, the RHSC during that year admitted 78.9% of respiratory infections compared to 9.4% at Ruchill and 11.7% at SGH (Table 8).

A comparison of total number of admissions and total number of children under study from October

COMPARISON OF TOTAL NUMBER OF ADMISSIONS FOR RESPIRATORY INFECTIONS AND TOTAL NUMBER OF CHILDREN UNDER STUDY ROYAL HOSPITAL FOR SICK CHILDREN, GLASGOW, 1985-86

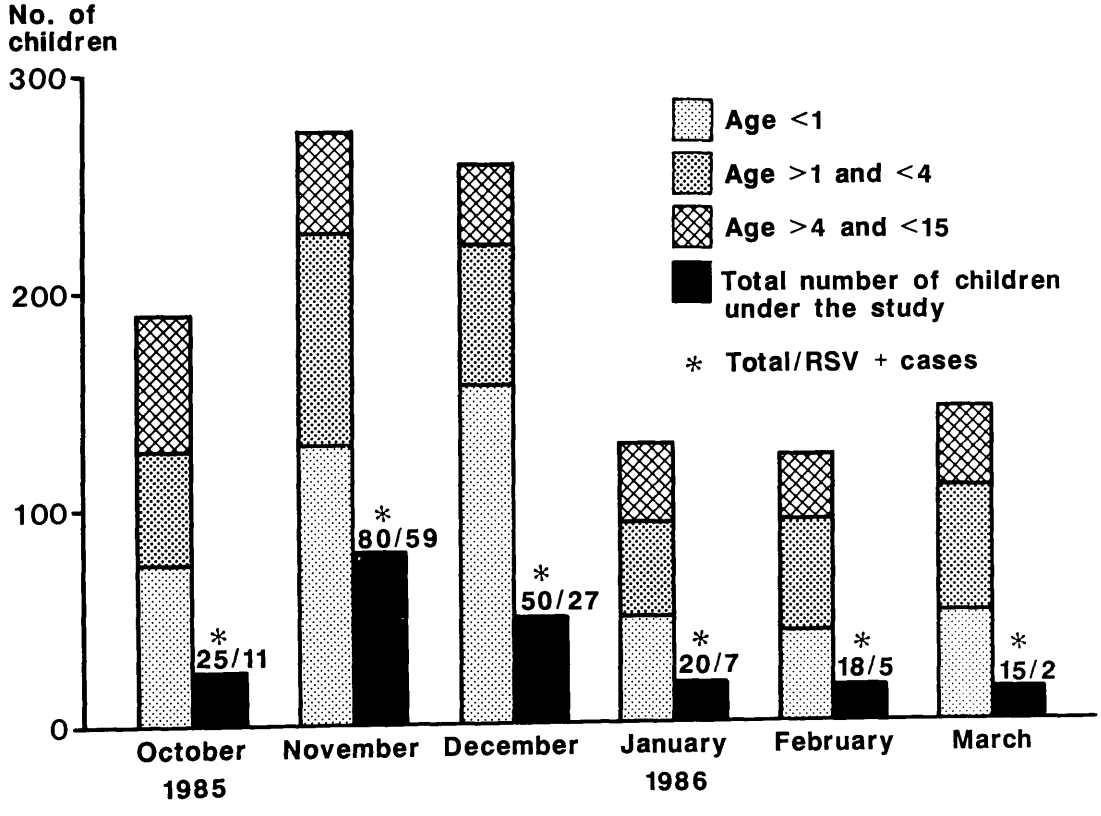


Figure 28

TABLE 7

Number of respiratory admissions in children less than 15 years at the Royal Hospital for Sick Children, Southern General and Ruchill Hospitals, Glasgow June 1985 to May 1986

Clinical presentation	RHSC (%)	SGH (%)	Ruchill (%)	Total
URTI	712 (72)	171 (17.3)	104 (10.5)	987
Bronchiolitis	393 (80)	47 (9.6)	49 (10.0)	489
Pneumonia	120 (86)	8 (5.8)	11 (7.9)	139
Chronic lung disease	678 (87)	76 (9.8)	24 (3.0)	778
Total	1903 (79.5)	302 (12.6)	188 (7.9)	2393

From: The Greater Glasgow Health Board, 1987

TABLE 8

Number of respiratory admissions in children less than 4 years at the Royal Hospital for Sick Children, Southern General and Ruchill Hospitals, Glasgow October 1985 to March 1986

Clinical presentation	RHSC (%)	SGH (%)	Ruchill (%)	Total
URTI	277 (84.0)	14 (4.2)	39 (11.8)	330
Bronchiolitis	324 (87.8)	2 (0.5)	43 (11.6)	369
Pneumonia	52 (53.0)	41 (41.8)	5 (5.0)	98
Chronic lung disease	215 (71.0)	72 (24.0)	16 (5.3)	303
Total	868 (78.9)	129 (11.7)	103 (9.4)	1100

From: The Greater Glasgow Health Board, 1987

TABLE 9

Total number and RSV positive cases in Glasgow area October 1985 to March 1986

	Area	No. children	No. RSV (+)
G1	City Centre	1	1
G2	City Centre	1	0
G3	Yorkhill	4	2
G4	Townhead	1	1
G5	Gorbals	0	0
G11	Broomhill/ Partick	3	1
G12	Kelvindale/ Kelvinside	2	1
G13	Knightswood/ Jordanhill	5	4
G14	Yoker	6	4
G15	Drumchapel	16	10
G20	Maryhill/ Ruchill	6	1
G21	Springburn/ Balornock/ Cowlairs	5	-
G22	High Possil	11	5
G23	Summerston	0	0
G31	Dennistoun/ Parkhead	6	4
G32	Carntyne/ Shettleston/ Carmyle/ Vernon/ Tollcross	6	1
G33	Garthamlock/ Robroyston/ Blackhill/ Riddrie/ Ruchazie	8	3

contd.....

TABLE 9 (contd)

	Area	No. children	No. RSV (+)
G34	Garthamlock/ Easterhouse	5	3
G40	Bridgeton/ Dalmarnock	3	1
G41	Dumbreck/ Pollokshields/ Shawlands	4	3
G42	Toryglen	6	6
G43	Pollokshaws/ Newlands	1	1
G44	Kings Park/ Cathcart	1	1
G45	Castlemilk	14	9
G41	Shieldhall	5	3
G52	Penilee/ Cardonald/ Craigton/ Mosspark	4	2
G53	Pollok/ Nitshill/ Darnley	1	1
G69	Baillieston/ Chryston/ Gartcosh	1	1
G71	Uddingston/ Bothwell	0	0
G72	Cambuslang/ Blantyre	6	2
G73	Rutherglen/ Burnside	4	1
Total		137	73

TABLE 10

Percentage of households with two or more indicators of deprivation

LOCAL GOVERNMENT DISTRICTS	
Glasgow City	28.8%
Clydebank	23.4%
Strathkelvin	12.5%
Eastwood	6.4%
Bearsden/Milngavie	5.8%
Scotland	19.6%
HEALTH BOARDS	
GGHB	25.1%
Lanarkshire	20.6%
Argyll & Clyde	20.3%
Tayside	19.8%
Ayr and Arran	18.7%
Lothian	17.2%
Grampian	15.6%

From: Greater Glasgow Health Board (GGHB): 10 year report, 1974-1983.

1985 to March 1986 is shown in figure 28. The majority of admissions (45%) were children under one year of age with the highest incidence of respiratory infections in November (30.7%) at the RHSC. From October 1985 to March 1986, 324 cases of bronchiolitis were admitted to RHSC, and 95 of these (who were not excluded for prior antibiotic treatment or selected with regard to the number of symptomatic days prior to admission) were included in this study. It was therefore possible to study 29% of all cases of bronchiolitis at RHSC during the study period. These children were among the 208 with respiratory infection that constituted the study group.

6.1.2 GEOGRAPHICAL LOCATION

From the total number of children under study 111 were found RSV positive, 5 adenovirus positive and 1 influenza A virus positive. The majority of patients admitted to the study came from Inner Glasgow, 137, Clydebank, 25, Strathkelvin, 13, Eastwood, 11, and Bearsden/Milngavie 7 cases.

The distribution of the total number and RSV positive cases in Glasgow is shown in Figure 29 and 30 respectively. In Inner Glasgow 73 out of 137 children were RSV positive. Most positive cases were well spread out in the City but higher numbers were seen in G15-Drumchapel (10), G45-Castlemilk (9) G42-Toryglen (6) and G22-High Possil (5). Post codes and place

TOTAL NUMBER AND RSV POSITIVE CHILDREN IN INNER GLASGOW ADMITTED TO THE ROYAL HOSPITAL FOR SICK CHILDREN, GLASGOW, 1985-1986

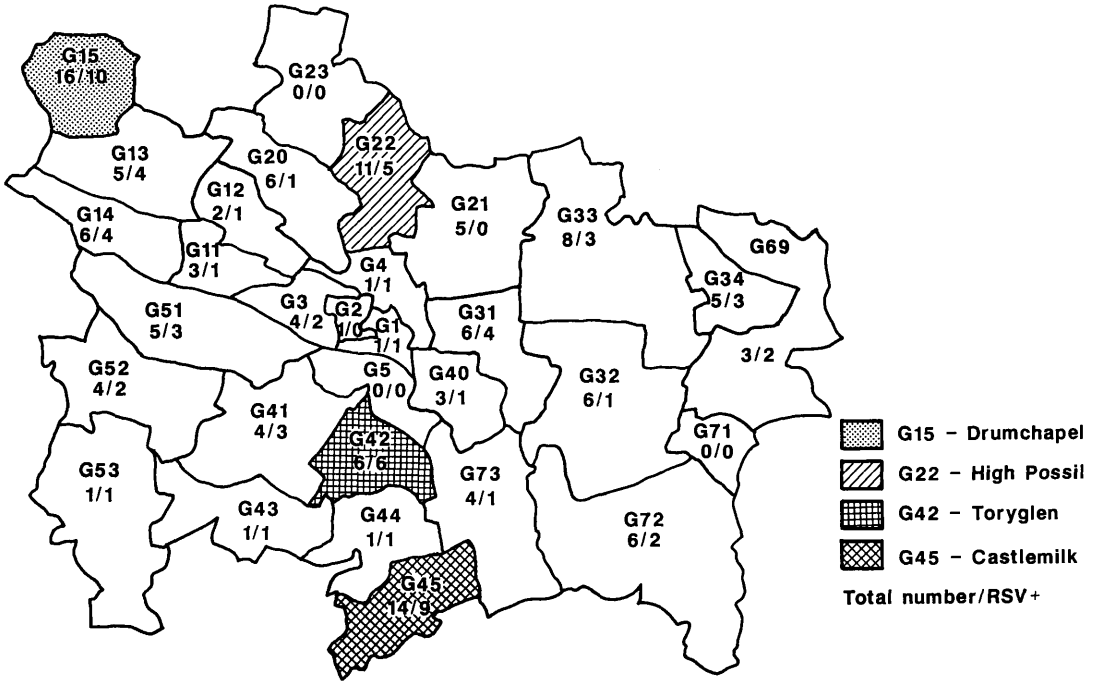


Figure 29

TOTAL NUMBER AND RSV POSITIVE CHILDREN IN OUTER GLASGOW ADMITTED TO THE ROYAL HOSPITAL FOR SICK CHILDREN, GLASGOW, 1985-1986

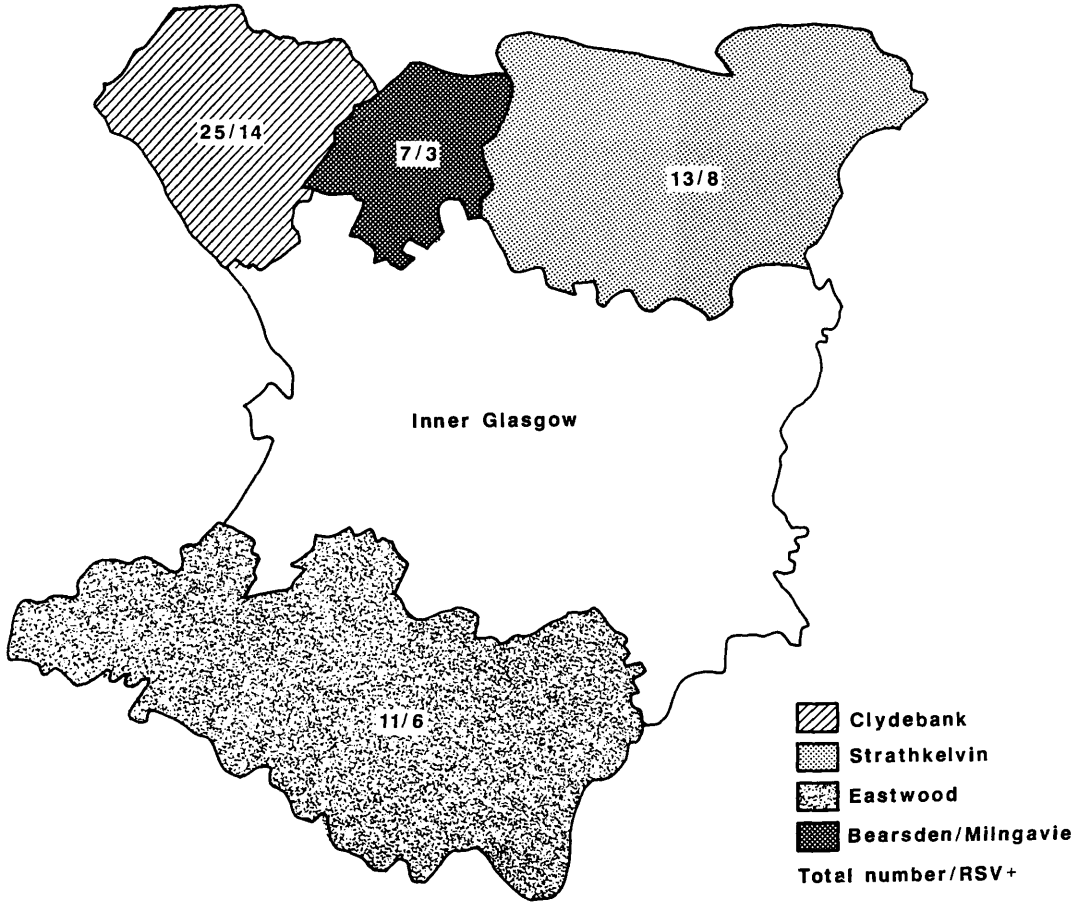


Figure 30

TABLE 11

Indicators of deprivation in each individual household in the Greater Glasgow Health Board Area

1. Head of household seeking work, permanently sick or disabled.

2. Occupancy: a measure of the rooms available in relation to the population resident in a household, taking into account the marital status, age and sex of each member of the household.

3. Head of household in socio-economic group: personal service, semi-skilled, skilled manual, agricultural.

4. Household with single parent family and dependent children.

5. Household with more than 3 dependent children.

6. Household contains only pensionable persons.

From: The Greater Glasgow Health Board: 10 year report, 1974-1983.

TABLE 12

Total number and RSV positive children with respiratory infection in the government districts and other places 1985-1986

Districts	No. cases %	RSV (+) %
Glasgow	137 (65.9)	73 (65.8)
Clydebank	25 (12.0)	14 (12.6)
Strathkelvin	13 (6.2)	8 (7.2)
Eastwood	11 (5.3)	6 (5.4)
Bearsden/Milngavie	7 (3.4)	3 (2.7)
Paisley, Lochwinnoch, Stewarton, Strathaven	5 (2.4)	4 (3.6)
Other parts of West of Scotland	10 (4.8)	3 (2.7)
Total	208	111

names are listed in Table 9. The proportion of households with two or more indicators of deprivation is shown in Table 10 taken from the Greater Glasgow Health Board (GGHB), 10 year report 1974-1983. It also shows that there is a higher proportion of multiply deprived households in the GGHB area than in any other Health Board in Scotland. There are 6 indicators as is shown in Table 11. In this study RSV infection was much greater in infants from Inner Glasgow particularly Drumchapel, Castlemilk, Toryglen, High Possil (65.8%) and Clydebank (12.6%) which have households with 2 or more indicators of deprivation.

The reverse situation was found in Bearsden/Milngavie and Eastwood where the proportion of RSV infections was 2.7% and 5.4% respectively (Table 12). Bearsden/Milngavie and Eastwood have a very small proportion of households with two or more indicators of deprivation (GGHB, 1983).

A summary of the proportion of RSV positive cases in the total number of children for each area is shown in Table 12.

There is a considerable variation in hospital admission rates for infants living in different parts of the GGHB. In the areas with high admission rates, morbidity may genuinely be higher with a greater incidence of infections and accidents than elsewhere. Alternatively children with a particular illness may be

TABLE 13

Age distribution of 202 children with respiratory infection due to respiratory syncytial virus, Royal Hospital for Sick Children, Glasgow 1985-1986

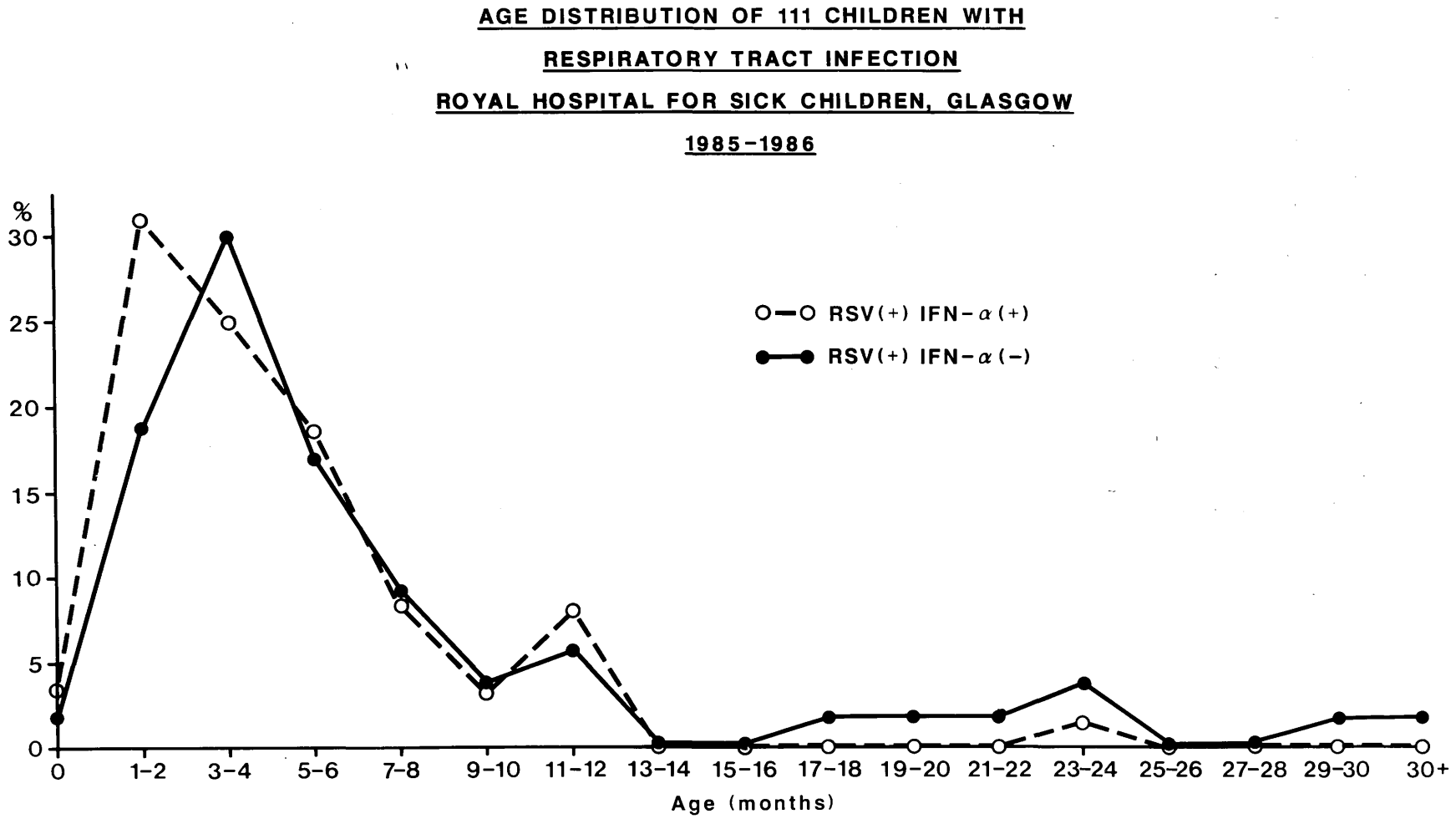
	Number of children	
Age (months)	RSV (+)(%)	RSV (-)(%)
<1	3 (2.7)	-
1-2	27 (24.3)	17 (18.7)
3-4	31 (27.9)	15 (16.5)
5-6	20 (18.0)	13 (14.3)
7-8	10 (9.0)	11 (12.1)
9-10	4 (3.6)	7 (7.7)
11-12	8 (7.2)	4 (4.4)
13-14	-	3 (3.3)
15-16	-	3 (3.3)
17-18	1 (0.9)	2 (2.2)
19-20	1 (0.9)	1 (1.1)
21-22	1 (0.9)	1 (1.1)
23-24	3 (2.7)	2 (2.2)
25-26	-	-
27-28	-	1 (1.1)
29-30	1 (0.9)	-
30+	1 (0.9)	11 (12.1)
Total	111	91

TABLE 14

Age distribution of 202 children with respiratory infection, Royal Hospital for Sick Children, Glasgow, 1985-1986

Age (months)	Number of children IFNα(+)(%)	IFNα(-)(%)
<1	2 (2.2)	1 (0.9)
1-2	25 (26.9)	20 (18.3)
3-4	17 (18.3)	29 (26.6)
5-6	18 (19.4)	15 (13.8)
7-8	8 (8.6)	13 (11.9)
9-10	8 (8.6)	3 (2.7)
11-12	6 (6.4)	6 (5.5)
13-14	1 (1.1)	2 (1.8)
15-16	3 (3.2)	-
17-18	-	3 (2.7)
19-20	-	2 (1.8)
21-22	2 (2.2)	-
23-24	1 (1.1)	4 (3.7)
25-26	-	-
27-28	-	-
29-30	-	1 (0.9)
30+	2 (2.2)	10 (9.2)
Total	93	109

Figure 31


AGE DISTRIBUTION OF 111 CHILDREN WITH
RESPIRATORY TRACT INFECTION
ROYAL HOSPITAL FOR SICK CHILDREN, GLASGOW
1985-1986
%
30
25
20
15
10
5
0
RSV(+) IFN-α(+)
RSV(+) IFN-α(-)
0
1-2
3-4
5-6
7-8
9-10
11-12
13-14
15-16
17-18
19-20
21-22
23-24
25-26
27-28
29-30
30+
Age (months)

more likely to be admitted to hospital if they are living in low income areas (GGHB, 1983).

6.1.3 AGE AND SEX

Table 13 shows the distribution of age in RSV positive and negative cases. A higher proportion of RSV positive cases was seen from 1 to 6 months of age and a sudden drop in the number of cases after 8 months was seen. In RSV negative cases the proportion of children from 1 to 8 months were quite similar and decreased steadily to 24 months with a second peak at 30 months.

The age distribution of the total number of children which presented with a positive or negative IFN-α response to infection is shown in Table 14. Alpha-interferon was present in a higher proportion of younger children (1-2 months), compared to IFN-α negative cases (3-4 months) and again gradually decreased with age.

The age distribution of RSV positive/IFN-α positive and RSV positive/IFN-α negative is shown in Figure 31. Three peaks of cases were identified in both groups: 1-4 months, 11-12 months and 23-24 months. Infants and children under one year of age comprise the major group of individuals with significant clinical illness consequent to RSV infection. In any year, a group of susceptible children are born subsequent to the previous RSV

TABLE 15

Sex distribution for age of 173 children with respiratory infection, Royal Hospital for Sick Children, Glasgow 1985-1986

Age	Male	%	Female	%
<1	2	(1.8)	1	(1.6)
1-2	32	(28.8)	13	(21.0)
3-4	30	(27.0)	17	(27.4)
5-6	22	(19.8)	12	(19.3)
7-8	15	(13.5)	6	(9.7)
9-10	5	(4.5)	6	(9.7)
11-12	5	(4.5)	7	(11.3)
Total	111		62	

TABLE 16

Sex distribution of 202 children with respiratory infection, Royal Hospital for Sick Children, Glasgow 1985-1986

	Male (%)	Female (%)	Ratio
RSV (+)	72 (64.9)	39 (35.1)	1.8
RSV (-)	53 (58.2)	38 (41.7)	1.4
Total	**125 (61.9)**	**77 (38.2)**	**1.3**

TABLE 17

Sex distribution of 111 children with respiratory infection, Royal Hospital for Sick Children, Glasgow 1985-1986

	Male (%)	Female (%)	Ratio
RSV (+) IFN-α (+)	40 (55.5)	25 (64.0)	1.6
RSV (+) IFN-α (-)	32 (44.4)	14 (36.0)	2.3
Total	72 (64.8)	39 (35.1)	1.8

TABLE 18

Sex distribution of 91 children with respiratory infection, Royal Hospital for Sick Children, Glasgow 1985-1986

	Male(%)	Female(%)	Ratio
RSV (-) IFN- α (+)	18 (32.0)	10 (28.6)	1.8
RSV (-) IFN- α (-)	38 (67.9)	25 (71.4)	1.5
Total	56 (61.5)	35 (38.5)	1.6

epidemic. The children are likely to be the cases admitted 12 months or 24 months later during a subsequent RSV epidemic. A peak for IFN-α positive cases was seen at 1-2 months of age compared to a peak at 3-4 months of age for IFN-α negative cases. Overall, the absence of IFN-α during RSV infection was more likely in patients over 6 months of age. IFN-α positivity in RSV positive cases was associated with a lower mean age (4.6 m) when compared to IFN-α negative cases (8.2 m), and this was statistically significant ($p<0.05$).

Analysis of sex distribution for age (Table 15) showed that more boys were admitted between 1 and 2 months than girls. The proportion in the other age groups was similar up to 8 years. Sex distribution of RSV positive and RSV negative cases is shown in Table 16 and shows a male predominance in both RSV positive cases (1.8:1 M/F) and in RSV negative cases (1.4:1 M/F). It would appear that whatever the infective cause boys are more likely to be admitted to hospital than girls. Sex distribution for RSV positive/IFN-α positive and RSV positive/IFN-α negative cases is shown in Table 17. The M/F ratio for both groups is still in favour of boys. Despite this M/F imbalance a similar proportion of males and females produce or fail to produce IFN-α in NPS during RSV infection. However, from this data girls are more likely to be

Figure 32

HOSPITAL STAY DURATION OF 111 CHILDREN WITH RESPIRATORY TRACT INFECTION

ROYAL HOSPITAL FOR SICK CHILDREN, GLASGOW

1985-1986

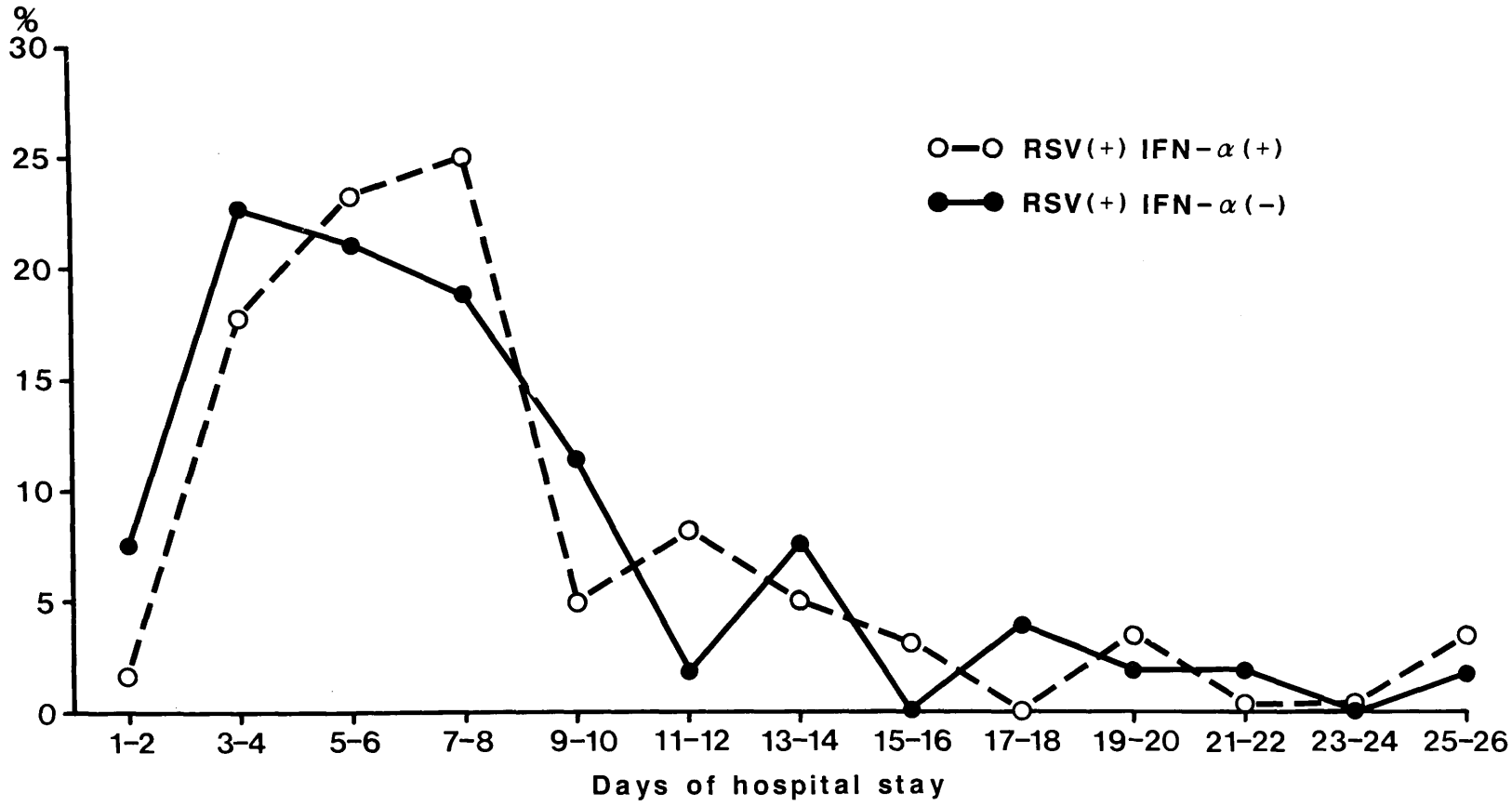


TABLE 19

Hospital stay duration of 202 children with respiratory infection, Royal Hospital for Sick Children, Glasgow 1985-1986

Hos/stay (days)	Number of children RSV (+)(%)	RSV (-)(%)
<5	28 (25.0)	40 (44.0)
5-8	50 (45.0)	18 (19.8)
9-12	15 (13.5)	15 (16.5)
13-16	9 (8.0)	6 (6.6)
17-20	5 (4.5)	2 (2.2)
21+	4 (3.6)	10 (11.0)
Total	111	91

chi squared = 19.9
$p<0.05$

IFN-α positive during RSV infection. The sex distribution of RSV negative cases is demonstrated in Table 18 where the M/F ratio is still in favour of boys, but girls producing less IFN-α than boys in these cases.

6.1.4 RESPIRATORY SYNCYTIAL VIRUS AND HOSPITAL STAY

Hospital stay duration in RSV positive and negative cases is shown in Table 19. The majority of children RSV positive were discharged after 5 or 8 days, whereas the majority of RSV negative patients were discharged in less than 5 days. The difference was statistically significant ($p<0.05$). The relationship between RSV positive cases and the presence or absence of IFN-α with respect to duration of hospital stay is shown in Figure 32.

The highest proportion of IFN-α negative cases were discharged at 3-4 days compared to 7-8 days for IFN-α positive patients. After 9-10 days there was no difference in the discharge pattern of cases. IFN-α positivity in RSV positive cases was associated with a longer hospital stay (8.6 days) compared (7.4 days) in IFN-α negative cases. However, there was no significant difference between these two groups when the Mann Whitney test was applied (Appendix 1).

6.1.5 ALPHA INTERFERON LEVELS

Mean levels and range of IFN-α positive NPS in 99 patients is shown in Table 20. Of the total

TABLE 20

Mean and range levels of alpha interferon by immunoradiometric assay in 208 children with viral respiratory infection, Royal Hospital for Sick Children, Glasgow 1985-1986

Virus	Mean IU/ml	Range IU/ml
RSV (+)	28.8	2-320
RSV (-)	37.3	2-120
Adeno	167.6	8-240
Influenza A	120.0	-

TABLE 21

A comparison of respiratory syncytial virus detection in tissue culture and monoclonal antibody techniques of 65 children with positive alpha interferon response, Royal Hospital for Sick Children, Glasgow 1985-1986

IFN-α IU/ml	Tissue culture (%)	mIFA (%)
2-20	9 (60.0)	43 (86.0)
>20	6 (40.0)	7 (14.0)
Total	15	50

number of patients (208) with suspected respiratory infection these 99 produced IFN-α in NPS with a mean level of 28.8 IU/ml (2-320 IU/ml) for RSV positive and 37.3 (2-120 IU/ml) for RSV negative cases. The five adenovirus positive cases had a mean level of IFN-α 167.6 (8-240 IU/ml) and one case with influenza A had a IFN-α level of 120 IU/ml. It is clear from other studies with other viruses that during RSV infection, IFN-α is produced in relatively low amounts in NPS. The levels of IFN-α for the RSV positive and RSV negative children is shown in Figure 33. It can be seen that RSV positivity was almost exclusively related to bronchiolitis whereas in RSV negative cases a broad spread among clinical cases was seen. Interferon positivity was found in 65% of children with RSV bronchiolitis patients compared to only 31% in children with bronchiolitis due to other unidentified causes.

Of the 111 positive NPS positive for RSV, 89 were detected by monoclonal immunofluorescence assay (mIFA) alone. The remainder (22) were positive by culture and by mIFA. No false negatives were found while using this mIFA. IFN-α levels in RSV positive NPS identified by tissue culture and mIFA is shown in Table 21. It was observed that NPS RSV positive by mIFA were more likely to be in the low positive range of 2-20 IU/ml IFN-α than culture positive samples (86%

ALPHA-INTERFERON LEVELS IN NASOPHARYNGEAL SECRETIONS OF FIVE GROUPS OF CHILDREN
ROYAL HOSPITAL FOR SICK CHILDREN, GLASGOW, 1985-1986

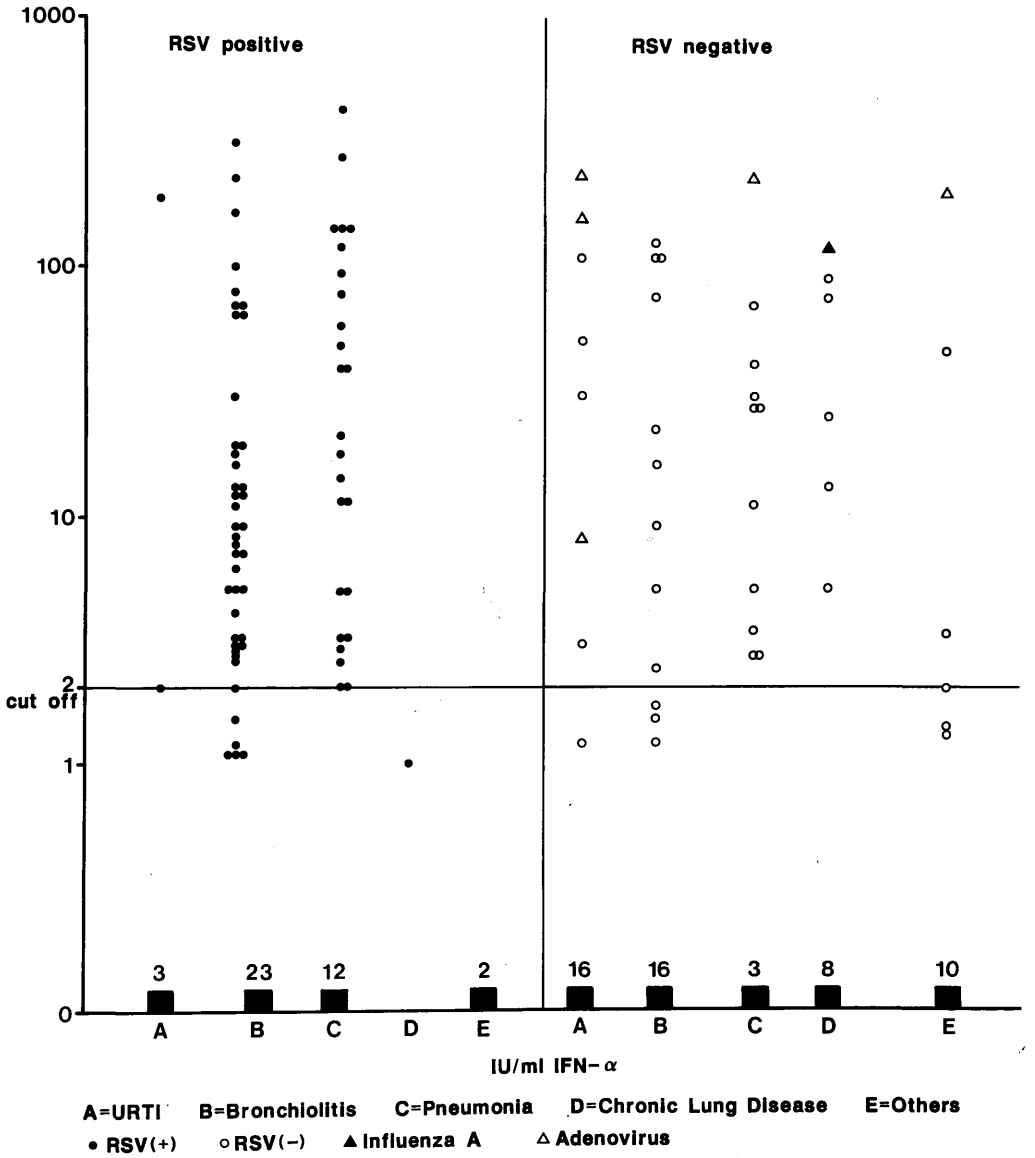


Figure 33

TABLE 22

Analysis of the total samples and cases

23A

Sample analysis	No.
Single samples	140
Two samples	35
Three samples	25
Four samples	4

23B

RSV positivity	No. (%)
Samples	182 (60.5)
Cases	111 (53.4)

23C

IFN-$\alpha \geqslant$ 2 IU/ml	No. (%)
Samples	142 (47.2)
Cases	93 (44.7)
Total samples	301
Total cases	202

TABLE 23

Result combinations for IFN-α and RSV in specimens and for cases

	No. RSV (+)(%)	No. RSV (-)(%)	Ratio
IFN-α (+)			(%)RSV(+)/RSV(-)
Specimens	103 (56.5)	38 (31.9)	1.77
Cases	65 (58.6)	28 (30.8)	1.90
IFN-α (-)			
Specimens	79 (43.4)	81 (68.1)	0.64
Cases	46 (41.4)	63 (69.2)	0.60

Total specimens (RSV+) = 182 Total specimens (RSV-) 119
Total cases (RSV+) = 111 Total cases (RSV-) 91

compared to 60%).

Nasopharyngeal secretions found RSV positive by culture demonstrated higher levels of IFN-α (>20 IU/ml) than mIFA (40% compared to I4%). As a positive tissue culture result is more likely to accompany a specimen at higher virus titre, an extrapolation of this data may suggest that a higher virus titre is also associated with higher IFN-α levels in NPS.

6.1.6 ANALYSIS OF SAMPLES AND CASES

The analysis of the total samples and cases is shown in Table 22. From the 202 children in the study 301 samples of nasopharyngeal secretions were obtained. The resulting combinations for IFN-α and RSV in specimens and for cases is shown in Table 23. When the proportion of RSV positive IFN-αpositive specimens and cases is compared with the proportion for RSV negative IFN-α positive specimens and cases; a ratio of RSV positive/RSV negative for IFN-αpositive specimens/cases can be obtained. This ratio was 1.77 for specimens and 1.90 for cases. For these ratios to remain similar, samples subsequent to the first sample analysed are likely to follow a similar result to that first obtained whether IFN-α positive or IFN-α negative. This would suggest that if IFN-α levels are linked with viral replication that individuals IFN-α negative at first sampling were in a final stage of illness, while those IFN-α positive were still in the

TABLE 24

Clinical presentation of 208 children with respiratory tract infection, Royal Hospital for Sick Children, Glasgow 1985-1986

	RSV (+)	RSV (-)	Adeno	Flu A	Total (%)
URTI	5	21	3	-	29 (14)
Bronchiolitis	66	29	-	-	95 (46)
Pneumonia	37	13	1	-	51 (24.5)
Chronic lung disease	1	13	-	1	15 (7.2)
Others	2	15	1	-	18 (8.6)
Total	111	91	5	1	208

chi squared = 54.5
$p<0.05$

URTI = Upper respiratory tract infection
RSV = Respiratory syncytial virus
Adeno = Adenovirus
Flu A = Influenza A

TABLE 25

Clinical presentation of 208 children with respiratory infection, Royal Hospital for Sick Children, Glasgow, 1985-1986

Clinical presentation	IFN-α (+)	IFN-α (-)
Total	99 (%)	109 (%)
Bronchiolitis	48 (48.5)	47 (43.1)
URTI	9 (9)	20 (18)
Pneumonia	34 (34.3)	17 (16)
Chronic lung disease	4 (4.0)	11 (10.1)
Others	4 (4.0)	14 (12.8)

chi squared = 18.23
$p<0.05$

TABLE 26

Clinical presentation of 202 children with respiratory infection, Royal Hospital for Sick Children, Glasgow 1985-1986

Clinical presentation	RSV positive		RSV negative	
	IFN-α(+)(%)	IFN-α(-)(%)	IFN-α(+)(%)	IFN-α(-)(%)
Total	65 (58.6)	46 (41.4)	28 (30.8)	63 (69.2)
Bronchiolitis	38 (58.5)	28 (60.9)	10 (35.7)	19 (30.2)
URTI	2 (3.1)	3 (6.5)	4 (14.3)	17 (27.0)
Pneumonia	25 (38.5)	12 (26.1)	8 (28.6)	5 (7.9)
Chronic lung disease	-	1 (2.2)	3 (10.7)	10 (15.9)
Others	-	2 (4.3)	3 (10.7)	12 (19.0)

chi squared = 1.88
$p > 0.05$

URTI = Upper respiratory tract infection

acute viral stage. This hypothesis may explain the higher proportion of infants RSV positive IFN-α negative discharged at 3-4 days compared to 7-8 days for IFN-α positive patients.

6.1.7 CLINICAL PARAMETERS

When clinical presentation of respiratory tract infection was analysed (Table 24), 95 children had bronchiolitis, 29 had URTI, pneumonia 51, chronic lung disease 15 and 18 had other symptoms such as diarrhoea, convulsions and congenital heart disease. Bronchiolitis was associated with the highest number of RSV positive cases and the difference between RSV positive and RSV negative cases was found to be significantly different ($p<0.05$). Table 25 presents data on the presence of IFN-α in the 208 children with respiratory infection. A statistical difference was found between IFN-α positive and negative cases ($p<0.05$) with the IFN-α positive cases predominantly related to pneumonia rather than URTI, bronchiolitis or chronic lung disease.

The relationship between RSV/IFN-α positivity and negativity in relation to clinical presentation is shown in Table 26. When these two groups were compared in a chi squared test for clinical presentation no significant difference was found ($p>0.05$).

Although 25/37 cases of RSV pneumonia were

TABLE 27

Maximum respiratory rate in 202 children with respiratory infection, Royal Hospital for Sick Children, Glasgow 1985-1986

Maximum respiratory rate (min)	IFN-α (+)	%	IFN-α (-)	%
Total	96		106	
<60	46	(47.9)	66	(62.3)
≥ 60	50	(52.1)	40	(37.7)

chi squared = 4.20
$p<0.05$

TABLE 28

A. Time to establish normal respiratory rate in 199 children with respiratory infection, Royal Hospital for Sick Children, Glasgow, 1985-1986

Time to establish normal respiratory rate (days)	IFN-α (+) (%)	IFN-α (-) (%)
Total	93	106
0-3	63 (67.7)	77 (72.6)
4-7	30 (32.3)	29 (27.4)

chi squared 5.03
p>0.05

B. Physiotherapy in 150 children with respiratory infection. Royal Hospital for Sick Children, Glasgow, 1985-1986

Physiotherapy (days)	IFN-α (+) (%)	IFN-α (-) (%)
Total	64	86
0-3	41 (64.0)	68 (79.0)
4-7	23 (36.0)	18 (21.0)

chi squared 4.33
p>0.05

C. Length of tube feeding in 200 children with respiratory infection, Royal Hospital for Sick Children, Glasgow, 1985-1986

Length of tube feeding (days)	IFN-α (+) (%)	IFN-α (-) (%)
Total	93	107
0-3	67 (72.0)	86 (80.4)
4-7	26 (28.0)	21 (19.6)

chi squared 1.93
p>0.05

IFN-α positive in NPS, 8/12 RSV negative cases were similarly positive for IFN-α. One of these 8 cases of IFN-α positive/RSV negative pneumonia cases was identified as adenovirus positive (Table 24). For IFN-α positive cases it would appear that other unidentified viral pathogens were responsible for these pneumonia cases.

Among the clinical parameters, maximum respiratory rate, time to establish normal respiratory rate, physiotherapy and length of tube feeding were evaluated statistically for an association with IFN-α regardless of aetiology. Maximum respiratory rate in children IFN-α positive and negative is shown in Table 27. A statistically significant value was achieved for this clinical parameter. Tables 28A, B and C, showed no significant difference between IFN-α positive and IFN-α negative cases, although a trend for worse clinical outcome was seen for physiotherapy and length of tube feeding.

The same parameters were similarly evaluated in RSV positive and negative children and results are shown in Tables 29 and 30. Other parameters were analysed and are reported later in this section. A respiratory rate equal or greater than 60/min was found in 59 of 180 children (Table 29A). A higher percentage of children was found in the RSV positive group (56.7%) compared to the RSV negative group

TABLE 29

A. Maximum respiratory rate in 180 children with respiratory infection, Glasgow 1985-1986

Maximum respiratory rate (min)	RSV (+)	(%)	RSV (-)	(%)
Total	104		76	
<60	45	(43.3)	46	(60.5)
$\geqslant 60$	59	(56.7)	30	(39.5)

chi squared = 18.16
$p<0.05$

B. Time to establish normal respiratory rate in 197 children with respiratory infection, Royal Hospital for Sick Children, Glasgow 1985-1986

Time to establish normal respiratory rate (days)	RSV (+)	(%)	RSV (-)	(%)
Total	109		88	
0-3	68	(62.4)	70	(79.5)
4-7	41	(37.6)	18	(20.5)

chi squared = 20.9
$p<0.05$

TABLE 30

A. Physiotherapy in 150 children with respiratory infection, Royal Hospital for Sick Children, Glasgow 1985-1986

Physiotherapy (days)	RSV (+) (%)	RSV (-) (%)
Total	81	69
0-3	53 (65.4)	56 (81.1)
4-7	28 (34.6)	13 (18.8)

chi squared = 17.33
$p<0.05$

B. Length of tube feeding in 200 children with respiratory infection, Royal Hospital for Sick Children, Glasgow 1985-1986

Length of tube feeding (days)	RSV (+) (%)	RSV (-) (%)
Total	111	89
0-3	81 (72.9)	72 (80.9)
4-7	30 (27.0)	17 (19.1)

chi squared = 13.48
$p<0.05$

(39.5%) and this was statistically significant at $p<0.05$. Time to establish normal respiratory rate was analysed in 197 children (Table 29B). Children RSV positive (37.6%) took 4-7 days to establish normal respiratory rate compared to 20.5% RSV negative over the same period ($p<0.05$).

Days in which physiotherapy was administered was evaluated in 150 children (Table 30A) and physiotherapy was required for a longer period in RSV positive cases (34.6%) compared to RSV negative cases (18.8%) ($p<0.05$).

Length of tube feeding in 200 children is shown in Table 30B. Tube feeding was applied for a longer period in those children RSV positive (27%) compared to RSV negative (19%) ($p<0.05$).

A similar analysis using the same clinical parameters was made for RSV positive cases and IFN-α status, i.e. IFN-α positive or negative. Results are shown in Tables 31 and 32. Maximum respiratory rate in 104 RSV positive children is shown in Table 31A. A maximum respiratory rate greater or equal than 60 breaths per minute was found in a higher proportion in RSV positive IFN-α positive patients (61.9%) compared to IFN-α negative patients (48.8%).

Time to establish normal respiratory rate was evaluated in 103 children (Table 31B). Children RSV positive IFN-α positive (43%) took 4-7 days to

TABLE 31

A. Maximum respiratory rate in 104 children with respiratory infection, Royal Hospital for Sick Children, Glasgow 1985-1986

Maximum respiratory rate (min)	RSV (+) IFN-α (+) (%)	RSV (+) IFN-α (-) (%)
Total	63	41
<60	24 (38.1)	21 (51.2)
≥ 60	39 (61.9)	20 (48.8)

chi squared = 1.74
$p > 0.05$

B. Time to establish normal respiratory rate in 103 children with respiratory infection, Royal Hospital for Sick Children, Glasgow, 1985-1986

Time to establish normal respiratory rate (days)	RSV (+) IFN-α (+) (%)	RSV (+) IFN-α (-) (%)
Total	63	40
0-3	36 (57.0)	26 (65.0)
4-7	27 (43.0)	14 (35.0)

chi squared = 0.63
$p > 0.05$

C. Physiotherapy in 52 children with respiratory infection, Royal Hospital for Sick Children, Glasgow 1985-1986

Physiotherapy (days)	RSV (+) IFN-α (+) (%)	RSV (+) IFN-α (-) (%)
Total	31	21
0-3	11 (35.5)	13 (62.0)
4-7	20 (64.5)	8 (38.0)

chi squared = 3.52
$p > 0.05$

TABLE 32

Length of tube feeding in 58 children with respiratory infection, Royal Hospital for Sick Children, Glasgow 1985-1986

Length of tube feeding (days)	RSV (+) IFNα(+) (%)	RSV (+) IFNα(-) (%)
Total	35 (60.3)	23 (39.7)
0-3	13 (37.1)	15 (65.2)
4-7	22 (62.9)	8 (34.8)

chi squared = 4.38
$p<0.05$

establish normal respiratory rate compared to RSV positive IFN-α negative patients (35%). Fifty two children RSV positive IFN-α positive (64.5%) required physiotherapy for a longer period (4-7 days) than children RSV positive IFN-α negative (38%) (Table 31C). Length of tube feeding in 58 children with respiratory infection is shown in Table 32. Tube feeding was applied for a longer period (4-7 days) to those children RSV positive IFN-αpositive (62.9%) compared to RSV positive IFN-α negative cases (34.8%). There was a significant difference between both groups for tube feeding but a statistically significant value was not achieved ($p>0.05$) with other parameters when IFN-α status was evaluated in RSV positive patients.

Although only one of the clinical parameters analysed was statistically significant for both RSV and IFN-α, there is a strong tendency linking severity of the RSV infection with the presence of IFN-α in NPS.

Other clinical parameters such as degree of bronchiolitis, maximum temperature, mechanical ventilation duration, maximum white cell count and lymphocytosis, intravenous fluids duration, maximum capillary CO2 did now show any significant correlation with RSV positive/negative, IFN-α positive/negative, RSV positive/IFN-αpositive and RSV positive/IFN-α negative status.

TABLE 33

Viral and bacterial agents in 208 children with respiratory infection, Royal Hospital for Sick Children, Glasgow, 1985-1986

Agents	Bronchiolitis (%)	URTI (%)	Pneumonia (%)	Chronic lung disease(%)	Other (%)
RSV	38(40.0)	3(10.3)	21(41.2)	1(6.6)	-
RSV + S.pneumoniae	11(11.5)	1(3.4)	5(9.8)	-	-
RSV + H.influenzae	11(11.5)	-	6(11.7)	-	-
RSV + coliforms	4(4.2)	1(3.4)	5(9.8)	-	2(11.1)
RSV + other Strep	1(1.0)	-	-	-	-
NK	1(1.0)	-	-	-	-
Adeno	-	1(3.4)	-	-	1(6.6)
Adeno + S.pneumoniae	-	1(3.4)	-	-	-
Adeno + H.influenzae	-	-	1(1.9)	-	-
Adeno + coliforms	-	1(3.4)	-	-	-
Adeno + other strep	-	-	-	-	-
NK	-	-	-	-	-
Influenza A	-	-	-	1(6.6)	-
S.pneumoniae	9(9.4)	5(17.2)	2(3.9)	3(20.0)	2(11.1)
H.influenzae	3(3.1)	5(17.2)	2(3.9)	-	2(11.1)
Coliforms	2(2.1)	-	-	2(13.3)	1(6.6)
No organisms	15(15.7)	9(31.0)	9(17.6)	8(53.3)	10(66.6)
Other Strep	-	1(3.4)	-	-	-
NK	-	1(3.4)	-	-	-
Total	95(45.7)	29(13.9)	51(24.5)	15(7.2)	18(8.6)

NK = not known

6.1.8 MICROBIOLOGY

Viral and bacterial agents in the total number of children is shown in Table 33. RSV was found alone in 40.0% of bronchiolitis which constituted the largest group with a known aetiology. The second group (41.2%) was RSV pneumonia where again RSV was found alone.

During the study period, RSV infections were associated equally with S.pneumoniae (11.5%) and H.influenzae (11.5%) of all cases of bronchiolitis. RSV and coliforms and other Streptococcus were found in a few cases, 4.2% and 1.0% respectively of bronchiolitis.

Streptococcus pneumoniae and H.influenzae were identified in a similar proportion of RSV associated pneumonia cases i.e. 13.5% and 16.2% respectively, or 9.8% and 11.7% of all cases of pneumonia.

S.pneumoniae was found alone in 9.4% of bronchiolitis cases while H.influenzae was found alone in only 3.1% of similar cases. In 15 cases of bronchiolitis (15.7%) no pathogens were identified. H.influenzae and Streptococcus pneumoniae were found in a similar proportion of URTI, pneumonia and other causes. Despite the fact, that adenovirus and influenza virus have been reported as causative agents of bronchiolitis, in the present study only 5 adenovirus and 1 influenza A (untyped) were detected.

Adenovirus isolates were confirmed by electron microscopy but no further experiments were performed to type these isolates.

Of the 5 adenoviruses, 3 were found in URTI cases. Influenza A virus was found in a case of chronic lung disease. Embryonated hen egg inoculation was performed in order to increase the isolation of influenza viruses in the population studied. Despite considerable effort this procedure was unrewarding in this study. The only influenza A virus detected in this study, during a poor influenza season, was by the method of monoclonal immunofluorescence detection in NPS deposits.

Insufficient serum samples were received in the study to make an adequate analysis of results. The study has concentrated on the detection of virus by direct methods in NPS and by tissue culture techniques. In a paediatric population such as this study it is likely that these techniques are in themselves sufficient to provide the best diagnostic opportunity for acute respiratory infections.

CHAPTER VII

7.1 DISCUSSION OF THE CLINICAL ASPECTS AND CONCLUSION OF THE THESIS

The present results support RSV as an important cause of epidemic respiratory disease in Glasgow particularly in young children. The RHSC has the highest annual admissions in Glasgow for respiratory infections in children (79.5%) compared to other paediatric departments such as Ruchill (7.9%) and SGH (12.6%) Table 7).

In comparing the observations from the Greater Glasgow Health Board to this study, a good correlation between peaks of respiratory infection and RSV cases during the winter season was noted at this laboratory (Figure 28). During that period, 29% of all bronchiolitis cases from the total number of those admitted at the RHSC together with other respiratory infections were studied for RSV and IFN-α status.

Although no direct comparison of risk by socioeconomic class has been formulated, the majority of RSV infections were higher in infants from socially deprived areas of Glasgow: Drumchapel, Toryglen, Castlemilk and High Possil (Figure 29). These areas have a higher proportion of low income families and

households with 2 or more indicators of deprivation (Table 10 and 11). The reverse situation was found in Bearsden/Milngavie and Eastwood areas which showed the lowest numbers of admission rates for RSV infection. In these latter areas the socioeconomic characteristics of the residents are better with 61% of the population belonging to social classes I or II (GGHB, 1983).

In the socially deprived areas, a higher admission rate can be explained by the following reasons. Firstly, the general practitioner or hospital doctor concerned may believe that hospital admission is more appropriate for children living in these areas. The home environment may not be conducive to rapid recovery; i.e. overcrowding, poor nutrition, smokey environment, dampness, poor parental understanding of problems and solutions. Secondly, it may be more usual for general practitioners in some areas to employ locums, some of whom may be unfamiliar with the practice and may therefore be more likely to seek hospital admission for the child. Thirdly, patients from certain areas may be more likely to present themselves directly to the hospital (GGHB, 1983).

Several host factors, including age, sex and the specific aetiology of acute respiratory infection may interact to determine the pathophysiological characteristics of a particular clinical illness. It has been reported that most infants hospitalized with

RSV infection were less than six months of age (Glezen et al., 1981). The data presented here describes the influence of age, sex and aetiological agent on the occurrence of respiratory infection in these children. A higher proportion of RSV cases was found in children from one to six months of age whereas a higher proportion of RSV negative cases were from those six to eight months of age (Table 13).

The present study is one of the few that examines IFN-α responses during the course of naturally occurring human viral disease.

Interferon responses in children examined in this study were found in a similar proportion of bronchiolitis cases, but were more likely to be found in cases of pneumonia ($p<0.05$) and less likely during URTI (Table 25). The only clinical finding for which IFN-α itself appears to be statistically significant was maximum respiratory rate (Table 27). Trends were seen in IFN-α positive cases for the remaining clinical parameters indicating 'worse clinical response' but these were not significant in statistical analysis (Tables 28A, B and C).

IFN-α levels in NPS secretions were determined in RSV positive and negative cases. IFN-α positivity was found in a higher proportion of very young children compared to IFN-α negative cases (Table 14). These findings differ from the one described by

McIntosh who did not find significant difference between children from 0-2 months and 6 and 8 months. Similarly, Hall et al., 1978, suggest that age alone did not appear to be a determining factor in the ability to produce IFN. The three peaks observed in both RSV positive IFN-α positive and RSV positive IFN-α negative groups (1-4, 11-12 and 23-24 months) (Figure 31) indicates that in any year a group of susceptible children are born subsequent to the previous RSV epidemic. With the passing of each RSV epidemic, the susceptible pool decreases with fewer admissions as age increases. This signals an 'acquired immune state' to 'clinical disease' or a removal of the anatomical or physiological factors that predispose younger infants to bronchiolitis.

A male predominance was found in this study (Table 15). Boys were admitted to hospital in higher numbers, whether RSV positive or negative (Table 16). For respiratory disease, it would appear that whatever the infective cause boys are more likely to be admitted to hospital than girls. When sex distribution was analysed with regard to IFN-α , a similar proportion of males and females produce or fail to produce IFN-α in NPS during RSV infection (Tables 17).

As girls are equally likely to become infected it is not clear why boys are seen to have a worse prognosis. This study has not highlighted any

clues as this enigma.

Children RSV positive were hospitalized for a longer period than RSV negative cases and the difference between them was statistically significant (Table 19). Similarly, duration of hospital stay related to IFN-α showed that RSV positive children stayed in hospital for a longer period compared with those IFN-α negative. Although the differences were not significant, these results suggest that children who presented with IFN-α had more severe disease requiring hospitalization for a longer period than those IFN-α negative cases (Figure 32).

Analysis of general and paediatric hospital discharge records for children for 1981 shows that there is a very considerable variation in hospital admission rates within the Greater Glasgow Health Board Area. Children from areas of high admission rates i.e. Maryhill/Ruchill area, tend to stay in hospital longer than children from low admission areas i.e. Eaglesham/Clarkston. The explanation proposed was that morbidity in children of areas of high admission rates was likely to be genuinely higher with a greater incidence of infections requiring hospital admission (GGHB, 1983).

The development and characteristics of IFN responses in RSV-bronchiolitis has not received much attention in the medical literature. RSV has

generally been considered to be a poor inducer of IFN during systemic or local infections in infants (Ray et al., 1967; Hall et al., 1978; McIntosh, 1978).

Hall et al., 1978 and McIntosh, 1978 observed that most children infected with parainfluenza or influenza viruses were able to produce IFN, whereas only a few children with RSV infection had detectable IFN titres in their nasal secretions. However,the present study using a sensitive immunoradiometric assay demonstrated IFN-α in NPS of a relatively high percentage of children (38%) with RSV infection. In RSV positive bronchiolitic patients, IFN-α was present in 65% compared to only 31% in bronchiolitic patients due to other causes (Figure 33).

Adenovirus infection in patients studied here was associated with IFN-α mean level of 167.8 IU/ml.

Data from in vivo studies of RSV as an inducer of IFN production are limited and conflicting. Evidence indicates that IFN in the nasal secretions is an important factor to the course and recovery from viral respiratory diseases (Moehring et al., 1971). Infants hospitalized with bronchiolitis or pneumonia due to RSV developed low or undetectable levels of IFN in the nasal secretions (McIntosh, 1978). It has been postulated that the lack of detecting levels of IFN in the nasal washes of infants may contribute to the severity of the disease and to the abundant and

prolonged shedding of RSV (Hall et al., 1978). However, it has been suggested that the dilution of IFN by watery secretions could be so great as to render it undetectable even in a normal child (Reid, 1982). The application of the 'Sucrosep IFN-α' test to NPS in this study enabled a comparison of four combinations for RSV and IFN status. As the ratios for (%) RSV positive/RSV negative cases for IFN-α positive specimens and cases (1.77 and 1.90) and for IFN-α negative and cases (0.64 and 0.60) were very close, it appears that when IFN-α is induced during RSV infection, its presence is consistently associated with the persistence of the virus. Also, that IFN-α is likely to be present at the first testing on admission in NPS of children who will produce IFN-α in the course of RSV infection. Of the total population RSV positive, 58.6% was found to be IFN-α positive compared to 41.4% for IFN-α negative cases. IFN-α was associated with RSV bronchiolitis in 46% of cases compared to 29% of RSV negative cases (Table 24). Interferon production is not an inevitable consequence of RSV infection, but when seen in a hospitalised case it is likely to persist while the case remains RSV positive. IFN-α production during RSV infection in infants has been demonstrated by IRMA assay in this study in a significant proportion of bronchiolitis and pneumonia cases. The detection of IFN, contrary to

other studies of similar cases appears to relate to the use of a sensitive and specific assay.

In this study a higher proportion of children RSV positive/IFN-α positive (61.9%) had a higher respiratory rate in contrast to RSV positive/IFN-α negative cases (Table 31A). A rapid respiratory rate usually indicates a good compensation by the patient and in severe cases the respiratory rate may decrease (Wohl, 1981). When the time in which normal respiratory rate was established, children RSV positive/IFN-α negative responded quicker than RSV positive/IFN-α positive cases (Table 31B).

Physiotherapy was administered for a longer period in RSV positive/IFN-α positive children (Table 31C) and tube feeding was also required for longer in this IFN-α positive, RSV positive group.

Despite the trends in various clinical parameters linking RSV disease severity with IFN-α positivity, the only clinical finding which was found to be statistically significant with the demonstration of circulating IFN-$\alpha$was the duration of tube feeding (Table 32). This is a good indicator of disease severity since tube feeding is always required in severe cases of bronchiolitis.

This data linking IFN-αpositivity with more serious disease contradicts other published data cited above. Although, it is logical that IFN-α

production should be beneficial and therefore shorten the viral illness, it is also logical to assume that in serious disease with persistent viral replication IFN-α induction will continue until the virus is eliminated. The results in this study would support this latter argument. Persistent IFN-α detection in RSV infection could then be seen as an indicator of worse prognosis and an possible future indication for antiviral therapy.

As expected, the present results also showed that RSV positive patients were statistically different from those RSV negative patients in all the clinical parameters analysed. RSV positive children presented higher respiratory rates and a higher proportion of them failed to established normal respiratory rates before 4 or 7 days (Tables 29A and 29B). Physiotherapy and length of tube feeding were applied for longer periods in those RSV positive patients (Table 30A and 30B).

The diagnosis of RSV in children has taken on an increasing importance as a result of the recognition of the frequency of infection in the paediatric population, and the possibility of treating severe infections with ribavirin and other antiviral drugs (Pothier et al., 1985). The direct immunofluorescence technique has proved to be a very useful diagnostic test for rapid detection of RSV antigen in NPS. The

significant advantage of immunofluorescence over isolation is related to the speed of reporting to the clinician allowing the prevention of nosocomial infections. The other major requirement for successful identification by immunofluorescence is high quality and specific reagents (Treuhaft et al., 1985).

The commercially available direct immunofluorescence reagent (Imagen) has been shown to have similar sensitivity and specificity characteristics to the conventional indirect immunofluorescence for the detection of RSV in nasopharyngeal secretions (Cheeseman et al., 1986). In comparing the time taken to carry out the tests, the direct mIFA is shorter, requiring one incubation step of 20 minutes before examination, compared with 2 hours for the indirect method.

The diagnosis of RSV was demonstrated by the direct immunofluoresence technique when found culture negative, in 89 cases. The remaining 22 cases were positive by culture and by fluorescence (Table 21). This reflected the high sensitivity of mIFA, compared to the Hep-2 cell culture system. It was observed that RSV positive NPS confirmed by fluorescence were more likely to be in the low positive IFN-α range of 2-20 IU/ml than culture positive samples which had a higher mean IFN-α level.

Bedside inoculation of cell culture tubes

with nasal washes has been reported to give excellent RSV recovery (Hall et al., 1975). Transport of the specimens at 4°C can also give the same results. In this medical centre, the specimens, as plain secretions, were sent without dilution in VTM and they arrived at the laboratory two or three hours after collection. This may have contributed to the low yield on culture.

In clinical situations NPS are usually obtained by a variety of personnel and transported by a routine messenger to the laboratory. Once there, additional delays may be encountered before inoculation in cell cultures. Cell cultures supporting RSV replication may themselves lose sensitivity with repeated passage and continual monitoring of this is required.

There are other factors in the recovery of RSV from clinical specimens by tissue culture which have to be considered. It has been observed that strains of RSV replicated optimally and produced cytopathology most rapidly when the Hep-2 cell monolayers were barely confluent at the time of inoculation (Treuhaft et al., 1985). Arens et al., 1986 found that the use of combinations of cell culture were more efficient than any individual cell type for isolation of RSV (Arens et al., 1986). In the present study, although specimens were inoculated in MRC-5 and

monkey kidney in addition to Hep- 2 cells, an improved the rate of isolation was not seen.

This study confirms the epidemic behaviour of RSV in the community. One hundred and eleven cases of RSV were diagnosed with the identification of only 5 cases of adenovirus and 1 case of influenza A. This study supports the view that RSV is the main aetiological agent for bronchiolitis, and does not require additional pathogens for disease to be severe.

To obtain a better isolation rate for influenza A, clinical specimens were inoculated into embryonated hen egg and tissue culture cells. 1985-1986 turned out to be a poor year for influenza isolates nationwide and this is reflected in this winter study. Influenza C virus is known, on the basis of serologic surveys, to be most prevalent among children. Attempts were made to isolate influenza C in the population studied without success. Influenza C is rarely associated with epidemics or outbreaks, and is rarely isolated: this is probably because the majority of Influenza C virus infections cause mild illness resembling the common cold, and tissue cultures are poorly sensitive to this virus.

Serological diagnosis was not always possible to achieve because it was particularly difficult to obtain convalescent serum samples from children several weeks after their acute illness had ended.

The role of bacteria in bronchiolitis has been debated for many years and its significance has not yet been established. One of the difficulties in hospital based studies has been the administration of antibiotics to many affected children before admission to hospital, thereby minimizing the usefulness of results obtained from throat and nasal secretions as indices of bacterial infection (Simpson et al., 1978).

Streptococcus pneumoniae was the bacterium most frequently isolated (Table 33) but its significance as a pathogen has to be evaluated on the basis that many children are pharyngeal carriers of this organism.

In conclusion, the present study has demonstrated that circulating IFN-α could be detected in a considerable number of infants with RSV-bronchiolitis if a highly sensitive assay such as the IRMA used here. This test was performed on NPS obtained from infants with acute respiratory disease and was found to be rapid, reproducible and specific. The assay was capable of being perfomed within 6 hours of specimen receipt and could be batched for testing. It could fit into diagnostic routines and provide information for both diagnosis in suspected virus disease and in management or as an indicator of the persistence of the virus and the effectiveness or otherwise of treatment regimes. The reproducibility of

the test was found to have a coefficient of variation of < 10% when tests were performed on the same day. Between assay precision was also acceptable, and provided the background for a routine test with minimal extra standardization.

The IFN-α IRMA assay in this study was able to measure very low levels of IFN-α (>0.2 IU/ml) and was not affected by the proteinaceous nature of respiratory secretions.

The specificity of the test was further proven by neutralisation-blocking assays which confirmed the presence of IFN-α in clinical specimens. Clearly one of the disadvantages of this test is the use of a labile radioactive tracer ^{125}I, reducing the shelf life of the reagents, requiring extra safety precautions and increasing the cost to potential users. However, the qualitative nature of radioimmunoassay gives precision to a method in which a cut-off of sensitivity can be measured allowing confidence at low levels of IFN detection. It is unlikely that the sensitivity of the system could be taken further without altering specificity and the value of measuring IFN-α < 0.2 IU/ml is also questionable.

In this study sensitivity considerations were very important as previous work had suggested that IFN was irregularly produced during RSV infection. The results of this study show that IFN-α induction during

RSV infection has been underestimated. However, the present study has confirmed predominantly low levels of IFN-α produced in the course of this disease. This method would appear to be superior to previous biological methods used and has provided more information on the value of IFN measurements. The IFN-α data is not conclusive, but this may have been improved if the clinical data was more critically kept in a prospective way compared, to the retrospective analysis that was made. Increased numbers in various clinical groups may have helped to achieve significance where trends were seen in multiple parameters.

This study does not give a clear indication where IFN fits into the pathogenesis of RSV infection and its associated bronchiolitis but has contributed to the knowledge of this infection and its significant impact on the infant below the age of one year. It is possible that IFN detection may have a place in the monitoring of a child's illness particularly if antiviral treatment is limited, by availability, cost, or toxicity, and when only selected patients can be treated.

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APPENDIX 1

1.1 Mean

The mean is a measure of location.

Sample mean = $\frac{\text{Sum of the observations}}{\text{Number of observations}}$

The mean of the 20 determinations of uninoculated fresh VTM was 93.2

2.1 Standard deviation

The most useful way to assess variation or variability in replicate observation.

$$s = \sqrt{\frac{\sum (\bar{x} - x_1)^2}{n-1}}$$

$\bar{x}$ = mean

xl = each value

n = number of determinations

The standard deviation of the 20 determinations of uninoculated fresh VTM was 9.

1.3 Coefficient of variation

It is calculated to express the standard deviation as a percentage of the mean analysis.

$$CV = \frac{SD}{x} \times 100$$

$$CV = \frac{9}{93.2} \times 100 = 9.6\%$$

1.4 95% Confidence limits

The 95% confidence limits are lower and upper boundary values within which the mean has a high probability of being bracketed or enclosed.

$$95\%\ CL = \bar{x} \pm t\ (SEM)$$

$\bar{x}$ = mean

t = Student t-statistic and is found by looking up the t-table

SEM = Standard error of the mean

$$SEM = s/\sqrt{n}$$

s = standard deviation

n = number of specimens

degrees of freedom = number of observation minus 1. So with n=20, there are 19 degrees of freedom.

t(19) = 2.093

The confidence limits were calculated for each point of the standard curve and they are shown in Table 3.

1.5 Equation of the regression line

Cubic fitted by least squares

The equation of the regression line was applied in order to determine the best fitting line of the standard curve through a series of points.

Equation y = log y x = log x

$$y = 4.79 + 0.394x + 0.118x^2 - 0.0112x^3$$

x	y	Pred y

0.1	101.8	104.4
1.0	132.6	120.3
4.0	243.4	252.9
16.0	642.0	701.1
64.0	2121.4	2142.4
256.0	6783.1	6034.6
1000.0	12297.5	12970.2

The best fit of a typical standard curve is shown in Figure 17.

1.6 Mann-Whitney U-test

It is a procedure for testing the null hypothesis of equal population location parameters.

Test statistics:

1. The two samples are combined and rank all sample observations from smallest to largest.
2. Assign tied observations the mean of the rank positions they would have occupied had there been no ties.
3. Sum the rank of the observations from population 1. If the location parameter of population 1 is smaller than the location parameter of population 2, it is expected that the sum of the ranks for population 1 to be smaller than the sum of the ranks for

population 2. Similarly, if the location parameter of population 1 is larger than the location parameter of population 2, it is expected the reverse to be true.

Depending on the null hypothesis, either a sufficiently small or a sufficiently large sum of ranks assigned to sample observations from population 1 causes us to reject the null hypothesis.

The test statistic is:

$$T = S - \frac{n1(n1+1)}{2}$$

where S is the sum of the ranks assigned to the sample observations from population 1.

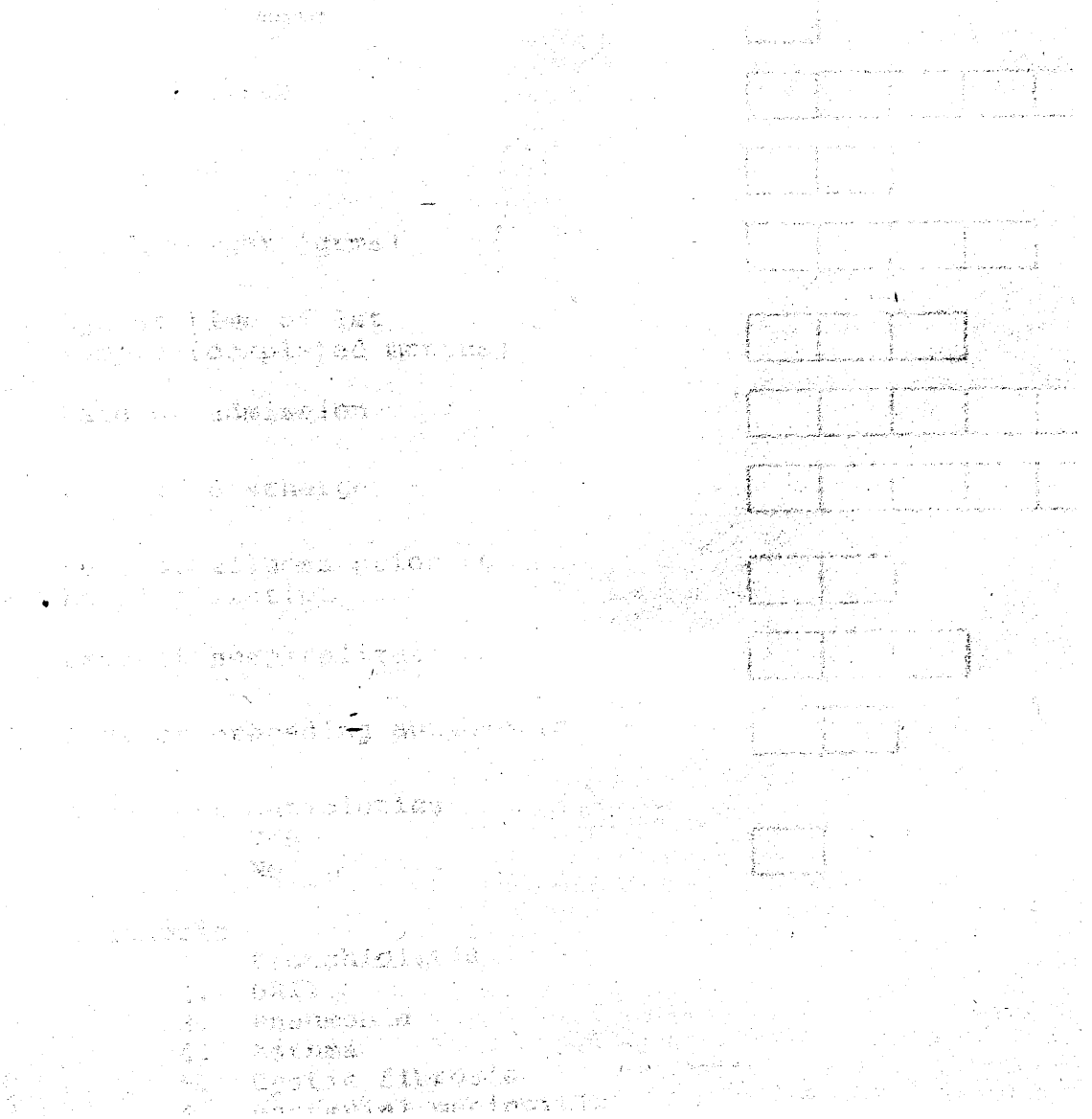
The null hypothesis is rejected if the computed T is less than the critical value of T using the table of quantiles of the Mann-Whitney test.

1.7 The chi-squared distribution

The type of hypothesis tested in chi-squared distribution is that the two criteria of classification are independent. In order to test the null hypothesis it must be compared with the frequencies that were actually observed with the frequencies which should have been observed if the null hypothesis were true. The analysis involves

the use of 2 x 2 contingency table.

The level of probability regarded as significant was 0.05.



INTERFERON STUDY

Hospital Number [| | | | | |] 6

Laboratory Number [| | | | | |] 12

Name .....

Ward .....

Sex 1. Male
2. Female [ ] 13

Date of Birth [| | | | | |] 19

Gestation [|] 21

Birth weight (grms) [| | | |] 25

Age at time of 1st sample (completed months) [| |] 28

Date of admission [| | | | | |] 34

Date of discharge [| | | | | |] 40

Days of illness prior to hospitalization [|] 42

Days of hospitalization [| |] 45

Days of preceding antibiotic [|] 47

Inpatient antibiotics
1. Yes
2. No [ ] 48

Diagnosis
1. Bronchiolitis
2. URTI
3. Pneumonia
4. Asthma
5. Cystic fibrosis
6. Bacterial meningitis
7. Aseptic meningitis
8. Bronchiolitis + URTI
9. Bronchiolitis + pneumonia
10. Bronchiolitis + congenital heart disease
11. Pneumonia + cystic fibrosis
12. Others [|] 50

Bronchiolitis
1. Mild
2. Moderate
3. Severe

[ ] 51

Oxygen Therapy
1. Yes
2. No

[ ] 52

Nasopharyngeal aspirate
1. Single
2. Two
3. Three
4. None

[ ] 53

Serum
1. Single
2. Two
3. None

[ ] 54

Urine
1. Single
2. Two
3. Three
4. None

[ ] 55

CSF
1. Single
2. Two
3. None

[ ] 56

Monoclonal fluorescence
Antibody for RSV
(Single specimen)
1. Respiratory Syncytial Virus positive
2. Respiratory Syncytial Virus negative

[ ] 57

Monoclonal fluorescence
Antibody for RSV
(Multiple specimen)

1. RSV positive in the first NPA and negative in the 2nd
2. RSV positive in the first and 2nd
3. RSV negative in the first NPA and positive in the 2nd
4. RSV negative in the first and 2nd
5. RSV positive in the first NPA and negative in the 2nd and 3rd
6. RSV positive in the first and 2nd and negative in the 3rd
7. RSV negative in the first and positive in the 2nd and 3rd
8. RSV negative in the first and 2nd and positive in the 3rd
9. RSV positive in the three specimens
10. RSV negative in the three specimens

[ ][ ] 59

Monoclonal fluorescence
Antibody for Flu A
(Single specimen)

1. Flu A positive
2. Flu A negative

☐ 60

Viral culture

1. RSV
2. Adeno
3. Flu A
4. Flu B
5. Enterovirus
6. Herpes
7. No virus isolated

☐ 61

Bacteriology

1. Streptococcus pneumoniae
2. Haemophilus influenzae
3. Coliforms
4. No organisms

☐ 62

Bacteriology (blood)

1. Streptococcus pneumoniae
2. Staphylococcus sp
3. Haemophilus influenzae
4. Coliforms
5. Neisseria meningitidis
6. No organisms

☐ 63

Interferon level
(Single specimen)

1. Positive
2. Negative

☐ 64

Interferon level
(Multiple specimen)

1. Positive in the first NPA and negative in the 2nd
2. Positive in the first and second
3. Negative in the first NPA and positive in the 2nd
4. Negative in the first and 2nd
5. Positive in the first and negative in the 2nd and 3rd
6. Positive in the first and 2nd and negative in the 3rd
7. Negative in the first and positive in the 2nd and 3rd
8. Negative in the first and 2nd and positive in the 3rd
9. Positive in the three specimens
10. Negative in the three specimens

☐☐ 66

Interferon level in the first sample ☐☐.☐ 70

Interferon level in the second sample ☐☐.☐ 74

Interferon level in the third sample ☐☐.☐ 78

Serum
Interferon level (first sample) 82

Interferon level (second sample) 86

Urine
Interferon level (first sample) 90

Interferon level (second sample) 94

Interferon level (third sample) 98

CSF
Interferon level (first sample) 102

Interferon level (second sample) 106

Serology by mIFA in the 1st serum (IgM)

1. RSV IgM positive
2. RSV IgM negative
3. Adeno IgM positive
4. Adeno IgM negative
5. CMV IgM positive
6. CMV IgM negative
7. Measles IgM positive
8. Measles IgM negative
9. Mumps IgM positive
10. Mumps IgM negative
11. Herpes IgM positive
12. Herpes IgM negative

108

Serology by mIFA in the first serum (IgG)

Respiratory syncytial virus 109

Adenovirus 110

Cytomegalovirus 111

Measles 112

Mumps 113

Herpes 114

All measures on this page are coded as follows

1. <8
2. 1:8
3. 1:32
4. 1:128
5. >1:256

Serology by mIFA in the 2nd serum (IgM)

1. RSV IgM positive
2. RSV IgM negative
3. Adeno IgM positive
4. Adeno IgM negative
5. CMV IgM positive
6. CMV IgM negative
7. Measles IgM positive
8. Measles IgM negative
9. Mumps IgM positive
10. Mumps IgM negative
11. Herpes IgM positive
12. Herpes IgM negative

[ ][ ] 116

Serology by mIFA in the 2nd serum (IgG)

Respiratory syncytial Virus [ ] 117

Adenovirus [ ] 118

CMV [ ] 119

Measles [ ] 120

Mumps [ ] 121

Herpes [ ] 122

All measures

1. <8
2. 1:8
3. 1:32
4. 1:128
5. >1:256

Complement fixation (1st serum)

Influenza A [ ] 123

Influenza B [ ] 124

Adenovirus Group [ ] 125

Mycoplasma pneumoniae [ ] 126

Psittacosis-LGV [ ] 127

Q-fever [ ] 128

Respiratory syncytial virus [ ] 129

Parainfluenza 1 (Sendai) [ ] 130

Herpes [ ] 131

Cytomegalovirus [ ] 132

Varicella-zoster [ ] 133

Mumps S [ ] 134

Mumps V [ ] 135

Measles [ ] 136

Leptospirae (gp-specific) [ ] 137

Complement fixation (2nd serum)

Influenza A		138
Influenza B		139
Adenovirus Group		140
Mycoplasma pneumoniae		141
Psittacosis-LGV		142
Q-fever		143
Respiratory syncytial virus		144
Parainfluenza 1 (Sendai)		145
Herpes		146
Cytomegalovirus		147
Varicella-zoster		148
Mumps S		149
Mumps V		150
Measles		151
Leptospirae (gp-specific)		152

All

1. <8
2. 1:8
3. 1:16
4. 1:32
5. 1:64
6. 1:128
7. 1:256
8. 1:512
9. 1:1024

Highest oxygen given [ ] 155

Time in O_2 (days) [ ] 158

Maximum Resp Rate (1 minute) [ ] 160

Time to establish normal resp rate (days) [ ] 162

Maximum temperature [ ] 165

Days of Physiotherapy (days) [ ] 167

Length of tube feeding (days) [ ] 170

Mechanical Ventilation Duration (days) [ ] 173

Maximum white cell count (x 10^9) [ ] 176

Maximum capillary CO_2 (mm Hg) [ ] 178

Social class (1,2,3,4,5) [ ] 179

Intravenous fluids duration (days) [ ] 181

Lymphocytosis

1. Yes
2. No

[ ] 182

APPENDIX 2

2.1 Preparation of rhesus kidney cells

A. Remove the supernatant medium from flasks of 75 cm2 with confluent monolayers of cells.

B. Wash the monolayer with 5 ml of phosphate buffered saline (PBS) Dulbecco's with phenol red, Ca and Mg free to reduce the number of these ions present. Remove it after 5 minutes.

C. Add 0.5 ml of a mixture trypsin/versene and incubate at 37°C for 5 minutes or until the cells can be seen dissociated.

D. Mix cells in 60 ml of medium 199 with Hanks' salts, with 0.35 g/l NaCO3, with glutamine and seed plastic culture tubes with 1 ml each.

E. Incubate at 37°C until used for viral isolation.

2.2 Preparation of MRC-5 and HEp-2 cells

A. Remove the supernatant medium from flasks of 75 cm2 with confluent monolayers of cells.

B. Wash the monolayer with 5 ml of phosphate buffered saline (PBS) Dulbecco's with phenol red, Ca and Mg free. Remove it after 5 minutes.

C. Add 0.5 ml of a mixture trypsin/versene and incubate at 37°C for 5 minutes or until the cells can be dissociated.

D. Mix cells in 30 ml of minimum essential medium (MEM) (Eagle) with Hanks' salts without L-Glutamine and seed plastic culture tubes with 1 ml each.

E. Incubate at 37°C until used for viral isolation.

2.3 Tissue culture growth media

2.3.1 Minimum essential medium (MEM)

MEM (Eagle) with Hanks' salts without L-glutamine	87 ml
Fetal calf serum (Mycoplasma and virus screened)	10 ml
Penicillin/Streptomycin (5.000 units/ml)	1 ml
Glutamine (200 mM/ml)	1 ml
Sodium bicarbonate (4.4%)	1 ml

Mix the above materials

2.3.2 Medium 199

Medium 199 with Hanks' salts with 0.35 gm/l NaHCO3 with L-glutamine	87 ml
Fetal calf serum (Mycoplasma and virus screened)	1 ml
Penicillin/Streptomycin	1 ml
Glutamine (200 mM/ml)	1 ml
Sodium bicarbonate (4.4%)	1 ml

Mix the above materials

2.4 Tissue culture maintenance medium

2.4.1 Minimum essential medium (MEM)

MEM (Eagle) with Hanks' salts and without L-glutamine	87 ml
Fetal calf serum (Mycoplasma and virus screened)	2 ml
Penicillin/Streptomycin (5.000 units/ml)	1 ml
Glutamine (200 mM/ml)	1 ml
Sodium bicarbonate (4.4%)	1 ml

Mix the above materials

2.4.2 Medium 199

Medium 199 with Hanks' with 0.35 g/l $NaHCO_3$	87 ml
Fetal calf serum (Mycoplasma and virus screened)	2 ml
Penicillin/Streptomycin (5.000 units/ml)	1 ml
Glutamine (200 mM/ml)	1 ml
Sodium bicarbonate (4.4%)	1 ml

Mix the above materials

2.5 Trypsin-EDTA solution

Contains 0.5 g Trypsin (1:250) and 0.2 g EDTA/l of Modified Puck's Saline A. This reagent is supplied ready for use.

2.6 Direct fluorescent monoclonal antibody test for RSV "Imagen" Boots Celltech Diagnostics Limited

Anti-RSV mouse monoclonal FITC conjugate.

One bottle containing 1.25 ml of Imagen RS virus reagent. This reagent is supplied ready for use and should be stored at 2-8°C in the dark and left at room temperature for 5 minutes before use.

2.7 Sucrosep IFN-α IRMA (Boots Celltech Diagnostic Limited

Standard preparations

A. Reconstitute the freeze-dried IFN-α standard of 1024 IU/ml using 3.0 ml of virus transport medium as a diluent.

B. Mix the bottle by gentle inversion and allow it to stand for approximately 15 minutes at room temperature.

C. Prepare a series of standards as follows:

IFN-α	Take volume	VTM(ul)	(IU/ml)
1024	stock	0	1024
1024	200	600	256
1024	100	1500	64
64	200	600	16
64	100	1500	4
4	200	600	1
-	0	1000	0

2.8 ^{125}I Monoclonal antibody to IFN-α (YOK 5/19)

This reagent is supplied ready for use.

2.9 Solid phase polyclonal antibody to IFN-α

This reagent is supplied ready for use.

2.10 Concentrated pre-wash buffer

A. Make up the contents of the bottle labelled as pre-wash buffer to 300 ml with distilled water.

B. The final solution may be stored in a closed container at 2-4°C and is stable for one month.

2.11 Sucrosep separation reagent

A. Make up the contents of the bottle labelled as sucrosep reagent to 600 ml with distilled water.

B. The final solution, colour coded light blue, may be stored in a closed container at 2-4°C and is stable for one month.

APPENDIX 3

3.1 Commercial sources of the reagents

3.1.1 Cells (HEp-2, MRC-5, rhesus monkey kidney)

Flow Laboratories Ltd

Rockingham Road

Uxbridge

Middlesex UB8 2UE

Tel: 0895 384512/2

Telex: 922083 Answerback FLOWWD

3.1.2 Medium 199, MEM, fetal calf serum, penicillin/streptomycin, glutamine, trypsin/versene, PBS, virus transport medium, Columbia agar (Oxoid) with horse blood, Columbia agar with horse blood, horse chocolate, Sabouraud Dextrose agar

Gibco Ltd

Trident House

P.O. Box 35

Renfrew Road

Paisley

Renfrewshire

Scotland PA3 4EF

Tel: 041-889 6100

Telex 777968/777029

3.1.3 Sodium bicarbonate

Wellcome Diagnostics

Temple Hill

Dartford

England DA1 5AH

Tel: Dartford (0322) 77711

Telegrams: Wellco Dartford

Telex: Wellco G 896113

3.1.4 Plastic tissue culture tubes

Labco Ltd

Marlow

England

3.1.5 Capillary tubes

Chance Propper Ltd

Smethwick

Warley

England

3.1.6 Bacitracin disks, Optochin disks

Oxoid Ltd,

England.

3.1.7 FITC monoclonal antibody for RS virus

Imagen reagent, Sucrosep IFN-α IRMA

Boots Celltech Diagnostics Ltd

Slough

Berkshire, England.

Tel: (0753) 821221

International: +44 753 821 221

Telex: 847805

3.1.8 Mucus traps

Mucus Extractor

Feltham,

Middlesex, England.

3.1.9 Api Strep for bacteria identification

Api Ltd

Basingstoke

England.